Immune modulation in gene therapy studies

Points to consider for Environmental Risk Assessment

FSF Aerts Kaya

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Erasmus MC
University Medical Center Rotterdam

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MANAGEMENT SAMENVATTING

Immuunmodulatie kan het milieurisico beinvloeden door twee mechanismen: langduriger vectorpersistentie zorgt voor een potentieel langduriger interactie met wildtype virussen en mogelijke recombinatie en kan de kans op en/of duur van shedding van de toegediende virale vectoren en de eventuele milieurisico's die daarmee samenhangen verhogen; immuun-gecompromiteerde individuen zijn gevoeliger voor infecties, met als gevolg een toegenomen kans op co-infectie met wildtype virussen. Huidige data uit dierstudies zijn onvoldoende om een voorspellende milieu risicoanalyse te doen voor de klinische situatie. Gebruik van immuunmodulatie in het klinische gentherapieveld moet daarom nauwgezet worden bijgehouden en data zouden makkelijk(er) toegankelijk moeten zijn om klinische protocollen vlug te kunnen bijwerken in antwoord op nieuwe ontwikkelingen.

Hier stellen wij het gebruik van een checklist voor om huidige milieurisico's binnen het gebruik van immuunmodulatie bij gentherapie onder de aandacht te brengen in de verwachting dat dit rapport zal kunnen dienen als een leidraad bij de risicobeoordeling en beleidsvorming bij gentherapiestudies.

Trefwoorden: Milieu Risicobeoordeling, Virale vector, Gentherapie, Immuunmodulatie

SUMMARY

Host immune responses play a major role in clearance of viral infections from the body, and may limit long-term expression and clinical efficacy of viral vectors. Methods to prevent these immune responses may also increase the risk for infections, recombination with wild type virus and affect biodistribution, persistence, shedding and transmission. The study described in this report was initiated to assess possible environmental risks associated with the use of immune modulation in combination with gene therapy and set up as a literature study, by performing PubMed searches for certain keywords, by interviewing experts and by attending selected meetings. Lack of availability of clinical data combining gene therapy and immune modulation and limited animal data warranted additional exploration of relevant non-gene therapy studies from closely related fields such as stem cell and organ transplantation, and vaccination studies with live attenuated vaccines.

Adenovirus-based (Ad) vectors induce a rapid immune response, resulting in the formation of neutralizing antibodies (NAb) and clearance by cytotoxic T-cells (CTL). Immune responses against Adeno-associated viruses (AAV) are considerably less robust, and largely dependent on the presence of helper virus, state of immune activation and the immunogenicity of the transgene. Clearance is mediated by a CTL and NAb response. Herpes Simplex Virus (HSV) vectors are highly immunogenic and induce a rapid activation of complement and a robust NAb and CTL response. Rapid capsid turnover of Lentivirus (LV) vectors prevents effective recognition of immunogenic epitopes by activated effector T-cells, but immune responses against LV may still occur in presence of an antigenic transgene. Clearance is predominantly NAb mediated.

Currently, a broad variety of immune modulatory agents is used in the (pre)clinical setting to prevent or dampen the immune response against viral vectors. Immune modulation with Cyclophosphamide (CY), an alkylating chemotherapeutic agent, was shown to result in a diminished formation of NAb and prolonged transgene expression when used with Ad, AAV and HSV vectors; CTLA4Ig is a fusion protein of the cytotoxic T lymphocyte antigen-4 (CTLA4, CD152) and an antibody and was able to decrease NAb and prolong transgene expression of Ad, AAV, and LV vectors, the use of Cyclosporin A (CsA), an immune suppressive agent, and Mycophenolate Mofetil (MMF), an inhibitor of purine biosynthesis in lymphocytes, showed variable results, but was in general only moderately or not effective. However, animal studies used different doses, different combinations, different time of administration, different routes of administration of the immune modulating agents, as well as different types and generations of viral vectors. In addition, differences in persistence, biodistribution and shedding were not systematically measured. Nevertheless, immune modulation appeared to affect biodistribution to some extent with more off-target transduction of the liver due to the potentially prolonged presence of vectors in the circulation in case of replication-deficient viral vectors, and a broader biodistribution pattern (ie to extratumoral tissue) for replication-competent oncolytic viral vectors.

Gene therapy animal models show a great discrepancy in the choice and timing of immune modulation and generally lack systematically obtained data on biodistribution and shedding. Both CY and CTLA4Ig can (transiently) prolong transgene expression, but increased risks for shedding or transmission were not reported in any study.

Future animal studies using immune modulation should include these data in their experimental setup to allow for a proper risk assessment.

Carefull reviewing of vaccinination studies with live attenuated viruses demonstrated that 1) albeit possible, adults are unlikely to transmit to other adults 2) high vaccine vector titers in pre-immune young children may increase the risk of transmission to other young children or even adults, 3) shedding and secondary or even tertiary transmission most often occur via direct contact with the site of vaccine inoculation of the vaccinee, 4) the risks of transmission depend on other environmental factors including frequent contact with excreta from other young children and the level of immune competence, and 5) transmission may occur due to breeches in precautionary measures despite written instructions.

Similarly, it is anticipated that the risks of recombination between recombinant viral vectors and related wild type viruses will be the highest in the pediatric pre-immune population. The risk of shedding is likely affected by additional handling, such as modification of vectors, affecting replication competentence and immune evasion mechanisms, immune modulation, the route of administration; the delivered viral vector load (single dose or/re-administration) and may remain unaltered in cases where expression of the transgene and transgenic proteins is increased, but duration of persistence of the viral vector remains unchanged.

Immune suppression or modulation can possibly affect the environmental risk by two mechanisms, i.e. longer persistence of the vector may increase the duration of interaction with wild type viruses and recombination and potentially increase the risk and/or duration of shedding of administered viral vectors and consequently the environmental risks; immune suppressed individuals are more susceptible to infections, thus increasing the chances of interaction with specific wild type viruses. Current data from animal studies are not sufficient to do a predictive environmental risk assessment for the clinical situation. Applications of immune modulation in the clinical gene therapy field therefore need to be followed carefully and access to data should be open and (more) readily accessible to be able to adjust clinical protocols quickly in response to new developments.

Finally, we propose the use of a checklist to assess current environmental risks in the use of immune modulation during gene therapy. This report is expected to provide guidance to risk assessors and regulatory officers as well as to applicants for a gene therapy licence.

Keywords:
Environmental Risk Assessment, Viral Vector, Gene Therapy, Immune modulation

1 INTRODUCTION

1.1 Rationale of the report

Host immune responses play a major role in the clearance of viral infections from the body. The induction of these immune responses are now being used to our advantage in the treatment of certain cancers, such as malignant melanoma, lung cancer, prostate cancer etc, by boosting the immune response against malignant cells, carrying viral antigens. However, for the treatment of monogenetic diseases, such as enzyme deficiencies, storage disorders, muscle diseases and immune deficiencies, the same (acquired) host immune response plays a crucial role as a determinant of long-term expression and clinical efficacy.

Viral gene products as well as transgene products, viral proteins, CpG DNA in plasmids and the transduced cells themselves all play an important role in inducing the host immune responses upon transfer into the targeted tissues. A key concern in the treatment of inherited protein or enzyme deficiencies by gene therapy is the risk for an immune response against the foreign therapeutic protein, which is distinct from the mutant protein produced by the patient. These immune responses may result in decreased efficacy and efficiency of gene therapy, leading to transient expression of therapeutic gene, non-efficient re-administration of the same vectors, and possibly (severe) side-effects in clinical trials.

As the potential success of gene therapy for chronic types of diseases depends fundamentally on long-term transgene expression to either cure or slow down the progression of a disease, immune modulation to dampen, eradicate or ideally prevent an immune response could increase the therapeutic benefits of gene therapy. Currently employed techniques to decrease immune responses include, among other, systemic immune suppression, blockade of co-stimulatory molecules, the use of anti-inflammatory cytokines, as well as (structural) modification of viral vectors. These methods to suppress the (acquired) immune response or to reduce the immunogenicity of vectors and transgenic proteins are used with the aim to have a more effective treatment. In cases where this leads to longer persistence of viral vectors the risk of interaction or recombination with wild type viruses and shedding subsequently increases, thereby possibly imposing an unintended environmental risk.

This study was initiated by the National Institute for Public Health and the Environment (RIVM)in a response to an increased demand of the governmental regulatory institutions 1) to assess the possible environmental risks associated with the use of immune modulation in combination with gene therapy; 2) to generate an overview of relevant data to assess new applications for future clinical trials involving immune modulation; and 3) to provide research groups with some background information on the immunology of gene therapy and the availability, possibilities, risks and advantages of current immune modulatory agents.

In this study, we evaluate the effects of immune modulation on the efficacy of gene therapy and provide an overall assessment of the environmental risk of recombination with wild type viruses and possible shedding. The study was designed as a literature study, using PubMed searches for various keywords, including immune suppression, immune modulation, gene therapy, immune reponses, tolerance and others. Additional information was obtained by attending relevant conferences and through personnal communications with experts in the field (for more information see Appendix D).

1.2 Lessons from vaccination and non-gene therapy transplantation studies

Data from preclinical and clinical gene therapy studies can be used for making risk assessments. However, these data are still limited and risk assessment could be further supported by making use of the experience from related fields, including (stem cell) transplantation and viral vaccination studies.

A major environmental safety concern for the use of a vector of viral origin for gene therapy is the potential spreading of the vector into the environment via excreta from the patient. This phenomenon is called shedding. Shedding of viral vectors does not *per se* result in person-to-person transmission and generally requires the presence of considerable levels of virus in multiple excreta and, more importantly, requires that treated individuals shed vector in an amount that is equal to or greater than a human infectious dose. In order to obtain better insight in the occurrence of viral spread to other persons viral vaccination studies were analysed for assessing the likelihood of horizontal transmission and furthermore to determine which factors are of influence.

Many of the agents currently tested for immune modulation in gene therapy, have been used as immune suppressive drugs in cancer treatment as well as in stem cell and solid organ transplantation to prevent GvHD and graft rejection. Reciprocally, these agents all carry the intrinsic risk of increasing the susceptibility to distinct infections, depending on the modes of action and duration of use. Patients with primary immune deficiencies and

with acquired immune deficiencies are also highly susceptible to a wide range of (viral) infections. Here, we give an overview of the different immune modulatory drugs currently used and their relation with the occurrence of viral infections and an outline of most common viral infections observed in immune compromised patients. Using these four categories of immune compromised patients as a "worst case" scenario for attracting virus infections, we will attempt to make a prediction of the risk of viral infections that might occur in gene therapy trials using immune modulation to prevent immune reactions against the viral vectors, viral proteins and transgenes. Based on this risk assessment, a risk estimation of recombination between wild type viruses and viral vectors, and possible shedding and transmission to health care personnel and relatives will be approximated and presented as a flow chart depicting relative risks of the use of immune modulation in combination with specific viral vectors. This flow chart can be used by governmental organizations, ethics committees and other advisory bodies to assess environmental risks for immune modulation gene therapy, but is also intended as a reference guide for clinicians during the planning, monitoring and reporting of new gene therapy trials.

1.3 Overview of this report

Data presented not only include gene therapy studies, but also all relevant non-gene therapy, clinical studies from related fields, including (stem cell) transplantation and viral vaccination studies, and all preclinical gene therapy studies using any type of immune modulation. This report consists of an introduction to the subject (chapter 1), the effects of immune modulation (chapter 2) and a list of points to consider when to assess the risks of immune modulation (chapter 3). Background information is provided in appendices: on immunogenicity of currently used viral vectors (appendix A), immune modulation currently used in the clinics (appendix B), lessons from vaccination studies with live attenuated vaccine strains (appendix C) and some lists of relevant literature and/or meetings (appendix D).

2 EFFECTS OF IMMUNE MODULATION

2.1 Persistence of viral vectors

In ongoing clinical trials, the use and choice of immune modulatory agents, is usually dictated by the underlying disease (genetic versus malignant disorder) and may be a requirement for the gene transfer procedure (e.g. hematopoietic stem cell transplantation). For genetic disorders this is done with the aim to obtain longlasting persistence of the transduced cells. For cancer there are increasingly more studies that want to make use of the host immune system in order to attack the cancer cells. Furthermore, in animal studies for genetic disorders, the focus has been on methods to decrease the host immune response against vector or transgene; whereas in preclinical studies for malignant disorders, the focus has been on methods to stimulate the specific anti-tumor response. To this purpose, a wide range of immune modulatory agents have been tested, including the use of different animal models, doses of the agents, administration regimens (before gene therapy, after gene therapy, multiple or single treatment), as well as combinations between immune modulatory agents. These data have been summarized in Table I Immune suppression used in clinical trials and animal studies with *ex vivo* gene transfer and Table II (A) Immune modulation used in animal studies using *in vivo* delivery of replicaton deficient vectors and (B) replication competent vectors, (C) Structural modications used in animal studies using *in vivo* delivery of replication competent vectors, (D) Immunodeficient animal studies using *in vivo* delivery of replication defective and (E) replication competent vectors. Table I shows immune suppression is used in order to provide space in the hematopoietic compartment for the transduced cells, which is especially needed when no growth advantage over non-transduced cells is to be expected. In Table II, it is shown that the most commonly used immune suppressive therapy was blockade of co-stimulatory signals, particularly by using CTLA4Ig, a fusion protein of the cytotoxic T lymphocyte antigen-4 (CTLA4, CD152) and an antibody, which blocks CD80 and CD86, followed by therapy with monoclonal antibodies. In addition, not only are almost all data concerning the effects of immune modulatory agents in combination with gene therapeutic procedures from animal studies, the majority of these studies involve either adenoviral (Ad) vectors or adeno-associated virus (AAV) vectors and an occasional study involves retroviral (RV) vectors. None of the studies used immune modulatory agents in combination with Herpes Simplex Virus (HSV)-based vectors, which is interesting considering the vast immune responses induced by HSV. This could be explained by the fact that HSV is mostly used in cancer therapy and that this property of HSV is taken as an advantage by enhancing the effect of the immune system in attacking the cancer cells. Therefore, the need to improve the efficacy of the therapy by using immune modulatory agents is more critically needed in cases where Ad and AAV-derived vectors are used for long-term gene correction. Upon administration, Ad induces a rapid and robust immune response due to binding to several blood components (complement, red blood cells, macrophages, etc) and clearance is mediated by a predominantly CD8+ CTL response. The immune response against AAV is generally moderate, but the slow uncoating of AAV capsid proteins allows for the induction of an anti-AAV-capsid CTL response and clearance of the vector by neutralizing antibodies. Clearance of AAV serotypes 1-9 from the circulation to <1% of the inoculated dose occurs typically within 48 hours[1]. In addition, widespread pre-existing immunity against both Ad and AAV necessitates the use of immune modulation, either through (transient) immune suppression or by vector modifications using stealth technology. Herpes virus and some retroviruses are known to pass from cell to cell by direct entry through and budding from the cell membrane of the cells. Viral antigen presentation is particularly abundant in cells infected by the latter type of viruses, marking these cells as 'infected' and rendering them susceptible to attack by cytotoxic T-cells. However, as in most cases RV and lentiviral (LV) vectors are used in *ex vivo* transduction procedures resulting in integration in the genome, from a shedding perspective these type of vectors become less relevant, since the vectors by itself are not expected to enter the circulation. When viral vectors such as Ad, RV/LV and HSV are used *in vivo*, immune suppressants affecting lymphocyte function or numbers, such as Cyclosporin, which specifically inhibits proliferation of T cells; ATG, which is used to deplete lymphocytes; and Campath, which is used to lyse CD52+ T cells, B cells and monocytes, could interfere here, and specifically prolong the presence of vectors, as well as mutant viruses, formed by recombination. In contrast, viruses that are predominantly cleared through formation of virus-specific neutralizing antibodies, such as AAV and Reovirus (Reo), could benefit from immune modulatory agents affecting B cell number and/or function or other interventions delaying or attenuating the development of neutralizing antibodies, eg Rituximab or Cyclophosphamide. Selective anti-B cell immune modulation could even enhance the effect of reovirus gene therapy, since the

oncolytic reovirus requires a certain level of functional T cells to ascertain killing of Reovirus-infected malignant cells.

2.2 Biodistribution

Biodistribution of the most commonly used gene therapy vectors in immune-competent animals is reviewed by Gonin and Gaillard (2004)[2]. Below you can find further information on (1) immune deficient animal models (Ad and Vaccinia Virus, VACV); (2) serotype-switching, circumventing pre-exisiting immunity (AAV); and (3) active immunomodulation (reovirus). Biodistribution (this paragraph) and shedding (paragraph 2.4) are discussed as separate entities here, but are clearly dependent on each other. Therefore some overlap in the discussions below cannot be avoided.

Adenovirus

Administration of replication-deficient human Ad5 (HAd5) based vectors to Severe Combined Immune Deficiency (SCID) mice resulted in whole body biodistribution, with the highest number of vector copy numbers in liver and spleen after intravenous injections, in prostate and liver after intraperitoneal injection and in draining lymph nodes after injection into paws and subcutaneous tissue[3]. Replication-deficient vectors based on non-human Ad serotypes, such as bovine Ad3 (BAd3) and porcine Ad3 (PAd3) can be used to circumvent pre-existing immunity and were found to distribute to the liver, spleen, lung, heart and kidney when intravenously injected into healthy FVB/n mice, with similar (PAd3) or even higher (BAd3) vector copy numbers than HAd5, detectable up to 16 days after intravenous injection[3].

In an athymic mouse xenograft tumor model, it was demonstrated that after a single intravenous injection of ONYX-015, 90% of the virus could be recovered from the liver within 3-6 hours, with 250 fold less infectious particles per gram tissue being found in the tumors. However, following tumorselective replication for 3 days, no infectious virus could be recovered from liver, while titers had increased 100-fold in tumor tissue, demonstrating the possibilities of systemic infusion in an immune deficient mouse model and the feasibility of this procedure for future clinical applications[3]. Evidence for viral replication within human tumor tissue and shedding of ONYX-015 into the circulation was found in patients receiving doses exceeding $2x10^{12}$ particles with 2.5 to 10 fold increased levels of viral genome detected in the plasma at 48 hrs after infusion in comparison to levels at 6 hrs. ONYX-015 has been used in many clinical trials sinds 1996, demonstrating safety, but limited efficacy. H101, another oncolytic adenovirus with an E1B-55 kDa deletion, was recently approved for marketing in China based on promising results in head and neck cancers[4]. After intratumoral injection into nasopharyngeal carcinomas, virus could be detected in blood, urine and the oropharynx. Combination treatment of H101 with cisplatin resulted despite leucopenia in an antibody response against H101 at day 22 after treatment[4].

Adeno-associated virus

AAV2 is the most commonly used serotype for gene therapy and has been used in a variety of clinical trials. Pre-existing neutralizing anti-AAV2 antibodies limit the efficacy of treatment and the use of alternative serotypes or pseudotyping may circumvent these limitations. Comparison of transduction efficiency and biodistribution of AAV-pseudotyped capsids 1, 5, 6 and 8 serotypes with single stranded (ss) or double stranded (ds) AAV2 after intravenous injection into immunocompetent male and female mice and imaging studies using luciferase as a marker to study biodistribution of AAV serotypes 1-9, demonstrated the widest biodistribution of AAV9, but the highest transduction efficiency of liver cells by AAV8 in mice, independent of the gender of the mice and the genomic structure of the vector; AAV4 vectors showed the greatest number of genome copies in lung, kidney and heart tissue; AAV5-pseudotyped vectors showed no appreciatable extra-hepatic gene expression; AAV6 vectors showed strong gene expression in the liver, the lower limbs skeletal muscle and heart muscle cells; both ssAAV1 and ssAAV8 vectors displayed significant gene expression in the lower abdominal area and gene transfer to the stromal cells of the gonads of female animals, but not in oocytes and gene transfer to offspring was not observed[1,5]. In C57Bl6 mice it was shown that upon iv injection, AAV6 vectors are shortly sequestered in liver (up to 72 hours) and spleen (largely cleared within 6 hours) and persist for up to 6 hours in serum, explaining the relatively efficient transduction of skeletal muscle cells[6]. Furthermore, it was shown that biodistribution of the several AAV serotypes is dependent on the route of delivery.

Retrovirus and Lentivirus

After intravenous injection of non-targeted replication-competent MLV-based vectors to tumor-bearing CB17 SCID mice, vector DNA was not only found intratumoral, but also distributed efficiently to most organs, appearing consecutively in spleen, liver and bone marrow

and to a lesser extent in the lung tissue, but not the brain[7]. Increasing PCR signal intensities and the detection of infectious viral particles in blood suggested continuous replication of MLV. In contrast, targeted MLV-based vectors, which are activated by matrix metalloproteinases (MMP) present in tumor cells, were found only in tumor sites, and showed no signs of extra-tumoral replication or biodistribution[7].

Most lentiviral vectors are based on the HIV-1 virus. However, the HIV-1 gp120 restricts transduction of HIV-1 vectors to CD4+ cells and limits its usefulness for other gene therapy applications. VSV-G is the most commonly used viral envelope protein used for LV pseudotyping, but other envelopes including rabies, MLV-amphotropic, Ebola, baculovirus, and measles virus envelopes have also been used to modify transduction patterns[8]. Pseudotyping with VSV-G results in a broad tropism and allows transduction of hematopoietic stem cells, brain, muscle and liver. Intraperitoneal injection of VSV-G pseudotyped LV encoding the immunomodulatory protein VIP in immunocompetent mice resulted in predominant biodistribution and transduction of the spleen, liver, adipose tissue and draining lymphnodes, but not kidney cells[9]. Intravenous injection of a VSV-G pseudotyped lentiviral vector encoding the adenosine deaminase (ADA) enzyme into neonatal ADA-deficient SCID mice resulted in primary transduction of liver and lung and low level transduction of peripheral blood leucocytes, thymus, spleen and bone marrow[10].

Vaccinia

Modified Vaccinia Virus Ankara (MVA) and MVA-based vectors were previously shown to be safe in immune deficient mice[11], SIV-infected monkeys[11] and HIV-infected humans[12], with clearance of all vector DNA, including the injection site, within 81 days in mice and 9 weeks in monkeys after intradermal injection. In normal, healthy C57Bl/J mice, MVA was detected 6 hours after intraperitoneal inoculation in almost all tissues studied, including the lungs, spleen, lymph nodes and ovaries and after subcutaneous injection predominantly in draining lymph nodes, although low level virus was detected in other organs as well, but was below detection levels at 48 hours after inoculation[13].

Reovirus

Wild-type Reovirus type 3 Dearing (RT3D), the most commonly used oncolytic reovirus strain, is non-pathogenic in healthy persons and infections are either asymptomatic or may result in mild respiratory or enteric symptoms. Clearance of the virus is predominantly mediated by neutralizing anti-reovirus antibodies. In a mouse tumor model, treatment with Cyclophosphamide was shown to significantly blunt the neutralizing antibody response against Reovirus and allow effective infection of tumor cells by RT3D. However, high doses of Cyclophosphamide abrogating any formation of neutralizing antibodies and complete absence of B-cells in B-cell knockout mice were associated with severe toxicity and distribution and replication of Reovirus in normal organs[14].

2.3 Recombination events in unmodified viruses and viral vectors

Mutation events such as removal or insertion of a nucleotide or a group of nucleotides (deletion or insertion mutants) are not uncommon during virus replication and are much more frequent in RNA than in DNA viruses[15]. In fact, all RNA viruses are thought to exist as mixtures of countless genetic variants with slightly different genetic and antigenic compositions. Recombination is brought about by the exchange and subsequent covalent linkage of genome fragments from a single gene or from two coinfecting related viruses[15] and does not generally occur among distantly or unrelated viruses. Recombination may give rise to a virus with hitherto unknown characteristics and may also give it a selective advantage over its relatives. More often though, the recombinant will have properties incompatible with survival[15] (personal communications with Ben Berkhout/Marco Schilham), for example the size of the recombinant genome may simply be too large for efficient packaging. With certain RNA viruses, such as influenza (orthomyxoviridae) and rotaviruses (reoviridae), in which the genome exists as separate fragments, simple exchange of genes may occur, a process known as gene reassortment. Such reassortant viruses have characteristics that differ from those of the parental viruses. The frequency of such gene exchanges may be very high, much higher than that of true recombination. Such genetic reassortment can extend the gene pool of the virus and allow the emergence of new and successful variants[15]. In addition, in at least some RNA viruses, such as influenza, very few or even a single nucleotide modification can turn a non-virulent strain into a virulent strain.

2.3.1 Recombination and mutation of wild type viruses

In general, RNA viruses are more prone to mutations due to lack of proofreading mechanisms, which cause a high mutation rate, and short replication times allowing RNA virus to rapidly adapt to a new environment. The ortho-

retroviruses, including the gammaretroviruses (MLV, HTLV-1 and HTLV-2) and lentiviruses (HIV-1, HIV-2 and SIV-2) replicate through a DNA intermediate, which is formed after virus entry and the process of reverse transcription. The formed double-stranded linear DNA is transported to the nucleus, where it is stably integrated into the cellular DNA of the host cell to form the provirus. Usually one DNA copy is generated from the two RNA genome copies that are present in the virion[16]. During reverse transcription, the RT enzyme can jump from one template strand to the other, thereby generating a hybrid transcript. If the two RNA templates are not identical, template switching (TS) can contribute to the overall retroviral mutation rate[16-18]. TS events can even take place between distantly related retroviruses, provided that the different viruses can cross-package the heterologous viral genomes[19]. It was calculated that the rate of retroviral recombination, during co-infection of viruses with distinct genomic RNAs, could reach up to 2% per kilobase per replication cycle, resulting in recombinant progeny in almost 50% of the mixed infections[16,17]. Furthermore, it was shown that recombination occurred only after the viral RNAs had been packaged into particles and that co-packaging of two different genomic RNAs as a heterodimer is a requirement for recombination[18]. Importantly, retroviral recombination does not occur during the initial infection, but takes place only after a second infection by the progeny of the initially infected cells. This unusual feature stems from the diploid nature of the virion genome and its mode of replication[18]. This could turn out to be relevant at later timepoints after gene therapy studies using retroviral vectors and immune modulation or in patients otherwise susceptible to superinfection with HIV. Nevertheless, the risks of recombination events are greatly diminished due to the absence of all viral genes in these vectors. Thus, orthoretroviral genomes are highly susceptible to the introduction of mutations, most of which are assumed to result from the action of the viral RT. In contrast, the foamy viruses are the most genetically stable viruses among the retrovirus family[20,21]. Analysis of recombination frequency of FV genomes revealed a 27% probability for a template switching event per 1 kb[20].

The DNA viruses are considerably less prone to mutations, as viral DNA polymerases have greater fidelity due to proofreading capability and viral genomes can remain stable under a variety of conditions. However, the existence of multiple (newly occurring) serotypes among the DNA viruses, eg adenoviruses and herpes viruses, suggests that some evolutionary mechanism is operative here as well. Homologous recombination of Ads was found to be restricted to closely related strains or serotypes within the same genus, in regions with the greatest homology[22]. For homologous recombination to occur, mammalian cells require an overlap of at least 200 bp[23]. Serotype specificity of Ad hexon-proteins is determined by hypervariable regions (HVR), which do not present sufficient sequency homology to allow recombination[24]. Illegitimate recombination resulting from the joining of two DNA sequence "hot spots" with limited homology in the HVR, as well as single base pair substitutions appears to play a role in the evolution of Ads[22]. Both illegitimate and homologous recombination events have been linked to species-diversion among the herpes viruses[25] and recombination rate can be very high (up to 40%) under experimental conditions[26,27], but also in HSV-1 natural populations, depending on local virus prevalence, host demography and rates of co-infection[28]. Biological properties of HSV-1 add to the likelihood of recombination due to high prevalence in the population, primary replication at muco-cutaneous sites, co-infection with more than one strain, rapid lytic cycles and latent persistence interrupted by reactivation and virus replication[29]. The presence of inverted repeats allows segment inversion through specific recombination and both intraspecific (same strain) and interspecific (distinct strains) recombination has been shown to occur, with coinfection as the most important prerequisite for successful recombination, followed by dose of virus, time interval between infection, distance between marker mutations, genetic homology, virulence and latency[29]. Poxviruses replicate in membrane-wrapped cytoplasmic structures called virosomes, which appear approximately 4-5 hours after infection[30]. Early virosomes display exponential growth lasting several hours, after which they migrate to the nuclear periphery. The rate of fusion between virosomes depends on the infecting particles, but even at high MOI (10 PFU/cell) approximately 20% of the virosomes never fuse. Furthermore, it was shown that upon fusion DNA mixes rather poorly, resulting in low likelihood of recombination even in the event of co-infection[30].

2.3.2 Recombination in replicating and replication-defective vectors

Vector/wild-type virus recombination events may arise if wild-type viruses related to the vector and the vector virus co-localize under favorable circumstances. Interchange, exchange or deletions of viral sequences in the vector may be repaired by homologous recombination or by reassortment. The presence of wild type virus may also give support in transcomplementation of deleted func-

tions. In addition, modifications on the vector may affect the risk for mutation and recombination. Also, during vector production in packaging cell lines, recombination may occur through transcomplementation of one or more missing genes. Testing for replication competent viral particles is therefore indicated.

First generation, second generation and helper-dependent adenoviral vectors

Transcomplementation of viral gene functions by a wild type Ad may result in the completion of a single life cycle, rather than in the generation of a replication-competent adenoviral (RCA) vector and therefore the impact on the patient and the environment is thought to be negligible. True recombination with a wild type Ad, resulting in RCA is possible and could result in prolonged viral replication and viral dissemination[31]. During production of first generation E1-deleted Ad vectors, the packaging cell line provides complementary functions and recombination and generation of RCAs is relatively easy. Some viruses cannot efficiently replicate when foreign sequences are inserted and most of the time only a moderate increase in size is allowed. For the development of helper-dependent Ad vectors, this knowledge was used to make the constructs safer: "stuffer" DNA, inserted to obtain a packageable genome size, does not prevent homologous recombination, but does result in genome sizes exceeding the packaging capacity of the Ad vectors[32]. In addition, wild type virus would have to supply multiple viral genes making the generation of RCA less likely. Instability of these viruses may result in loss of both the inserted sequences and sequences from the viral backbone, especially in cases of duplication of a particular sequence. The risks for recombination are largely dependent on the genetic stability of the wild-type or parental viruses. The possible consequences of these RCAs depend on the new tropism and virulence, as well as the presence or absence of a functional transgene.

Adeno-associated vectors

AAV is naturally replication incompetent and requires the presence of helper virus to complete its life cycle. Vectors based on AAV are designed to be replication-deficient and lack the *rep* and *cap* genes. It was previously shown in animal studies that replication-competent AAV can be generated, but this requires sequential infections with wild type AAV, in addition to the helper virus, and makes it highly improbable to occur in either cell culture or a clinical setting[31]. Since AAV is non-pathogenic and most adults have neutralizing antibodies against AAV, it is unlikely that recombination and generation of replication-competent AAV would result in transmission and human disease. The most likely scenario would be that the vector might cause a respiratory tract infection, although the total of effects may be dependent on the transgene[31].

Retroviral and lentiviral vectors

Wild type murine retroviruses are not known to infect humans and are not associated with any known human disease, possibly due to the rapidly induced innate immune response and inactivation mediated by human complement. The murine retroviruses are rendered replication incompetent by removal of the *gag*, *pol* and *env* genes and data from cell-culture studies suggest that the probability of RCR generation is, although theoretically present, low. Recently, replication-competent retroviral (RCR) vectors, based on MLV, have been modified for cancer treatment[33,34]. These RCRs have a reduction in replication efficiency due to the expression of heterologous genes, which require the elongation of the viral genome beyond its natural size and, similar to other RVs, are prone to mutations[35]. Deletions of the inserted foreign genes may give rise to replication-competent virus with a growth advantage due to increased replication efficiency, and with a resistance to subsequent superinfection with the parental vector[36]. Furthermore, it was shown that the genomic stability of the RCRs is also dependent on the host cell[37]. In the third generation self-inactivating (SIN) lentiviral vectors, also the essential genes for replication, *tat* and *rev*, and (parts of) the U3 region of the 3' long terminal repeat (LTR) are deleted, resulting in replication-deficient vectors. This minimizes the risk that replication competent lentivirus (RCL) will occur. But again, recombination with the necessary viral genes derived from the packaging cell line, may theoretically result in the regeneration of RCL vectors. These might, when administered to humans, disseminate widely throughout the body, due to the absence of complement responses against LV, in contrast to the early MLV vectors[31]. However, in an earlier advise (CGM/090331-03) from the Netherlands Commission on Genetic Modification (COGEM), the organization considered the chances on the occurrence of RCL negligible in third generation SIN lentiviral vectors[38]. Recombination *in vivo* is highly unlikely, unless the patient is a carrier of HIV. In these cases, the likelihood of recombination between the current type of lentiviral vectors and HIV is unknown, but such an event may not substantially affect the degree of pathology of the HIV disease (personal communication

with Ben Berkhout), unless there is other interference attributable to transgene expression.

Vaccinia vectors

Vaccinia virus is a replication-competent, attenuated pox virus with oncolytic capacity. It causes minor disease in healthy persons, but may lead to severe disease and encephalitis in immune deficient patients. Oncolytic Vaccinia vectors carrying specific transgenes become attenuated when the transgenes are inserted in non-essential genes, such as the thymidine kinase gene and display restricted tumor-specific replication. Insertion in the TK gene appears to decrease the side effects of vector administration related to central nervous system involvement. Recombination with wild type Vaccinia or other poxviruses has been thought to be highly unlikely[30,31].

2.3.3 Recombination of non-human/artificial vectors

Non-viral, plasmid-based gene therapy is the most commonly used type of non-infectious gene therapy. Currently, some clinical trials for peripheral vascular disease, and certain malignancies, such as head and neck cancer and melanoma are underway and certain DNA-based vaccines are being developed. However, the efficiency of gene transfer of naked DNA or DNA transfer through liposomes is low in comparison to virus-mediated transfer. Thus, plasmid-based gene therapy is relatively safe if standard precautionary measures are used, such as disinfection with 10% bleach, which eliminates any measurable DNA spill on surfaces. Spread from person to person or animal to animal appears unlikely, as the vectors do not replicate[31]. Vertical transmission or transfection of gonadal tissue in mice was never observed. Importantly, studies also suggest that there is no potential for replication due to recombination with any pathogen[31].

2.3.4 Effects of immune modulation on recombination risk

Immune modulation can affect the risk of recombination at at least two different levels, i.e. 1) immune suppressed individuals may be more at risk for reactivation of latent infections and primary infections with certain viruses, and 2) reduced immune responses against the viral vector may allow for prolonged persistence and affect biodistribution.

Adenoviruses are endemic and most adults have cross-reacting neutralizing immunity against multiple serotypes. However, immune suppressed individuals, especially children, are particularly vulnerable to both reactivation and primary infection with wild type Ad. The recombination risk therefore is dependent on the patient age, immune status, serotype of the vector and the type of Ad vector (first generation, second generation, HD-Ad).

AAV are non-pathogenic and naturally replication-deficient and most adults have cross-reacting neutralizing antibodies against AAV. Recombination of AAV would require the simultaneous presence of the AAV vector, a wild type AAV and the presence of a helper virus, such as HSV or Ad to provide the complementary functions. In children with acute respiratory infections coinfection of Ad and AAV was found to occur in 2% of sampled tissues[39]. AAV infections are not increased or more pathogenic in immune suppressed individuals, and recombination therefore seems highly unlikely.

Oncolytic vaccinia has been used in combination with several immune modulatory agents, including cyclophosphamide and rapamycin, resulting in increased intratumoral viral replication[40]. This could potentially increase the risk for recombination between viral particles, as it was shown to be dependent on the amount of infectious particles present during coinfection[30]. However, recombination rates are also inversely dependent on the time between virosome appearance and fusion[30]. Due to the eradication of variola in the human population, possible recombination events can only occur with zoonotic non-variola orthopoxviruses. Recombination risks of different vaccinia strains are therefore highly unlikely and can be relatively easily prevented by restricting access to possible carriers.

2.4 Shedding

Shedding is defined as the dissemination of a viral vector into the environment via excreta from a patient treated with gene therapy. Recently an inventory of shedding data from clinical trials was published by Schenk-Braat *et al*[41]. They showed that shedding of vectors occurs in practice and is mainly determined by the type of vector and the route of vector administration. The risks associated with shedding depend on multiple factors, including the pathogenicity of the viral vector, whether the vector is replication deficient or competent, the immunogenicity of the vector, the occurrence of pre-existing neutralizing antibodies in the healthy population, and the possibly toxicity of the transgene.

Adenovirus

Shedding of adenovirus may occur during administration from the site of the injection or after administration through droplets generated by coughing or sneezing, if the vector is administered as an aerosol into the lung,

or by fecal/oral contact, if the virus affects the gastrointestinal system. It is known from vaccination studies (see Appendix C), that shedding of Adenovirus vaccine strains from vaccinees occurs and that transmission may take place to secondary persons after close contact[42]. After vaccination of military recruits with live Ad4 or Ad7 vaccine, it was shown that fecal shedding occurred in at least 30% of the vaccinees, depending on the strain and could last from day 7-21 after vaccination[43]. The current Ad4 and Ad7 vaccines are contraindicated for individuals who are immune suppressed or those who have immune-compromised partners due to possible risks of increased shedding of replicating Ad[44].

In a recent clinical trial with a replication-defective Ad-CMV-p53 for treatment of esophageal cancer, it was shown that local tumor injection could result in significant shedding through the gastrointestinal tract, with almost 30% of stool samples and 13% of gargling samples positive for adenoviral DNA fragments for up to 12 days[45]. A recent overview on biodistribution and shedding data of the non-replicating vector HAd5 showed that both biodistribution and sites of shedding were dependent on route of administration, but that shedding via semen was unlikely[46].

Adeno-associated virus

In a recent trial for lipoprotein lipase deficiency with an AAV1 pseudotyped vector, vector sequences could be transiently detected after intramuscular injection in serum, saliva, urine, semen, and muscle biopsies using a sensitive quantitative PCR[47]. The highest vector concentrations were detected in the serum, with a rapid clearance by 1–2 logs per week. Urine was cleared from vector sequences at 1 week after treatment in patients treated with the lower dose of $1x10^{11}$ gc/kg AAV1-LPL^{S447X} and only very low levels (at maximum 25–58 copies/µg of DNA) were detected in the semen for short periods of time. Persistent presence of high levels of vector sequences was only detected in injected muscle. Leakage of vector from the injection site was limited and the risk for germline transmission was considered extremely low[47]. In a rabbit model, the risk of germline transmission of AAV2 was found to be dependent on the route of vector administration, with intravascular delivery of vector leading to dissemination in semen, but local injection in muscle not and infectious particles in semen could be detected up to a maximum of 4 days after injection and not thereafter[48]. Similarly, AAV2 sequences were detected in semen of AAV2-fIX vector recipients[49]. Data from vasectomized rabbits, treated with AAV2 or AAV8, however, suggest that not the germ cells, but the seminal fluid was the source of vector sequences and in the absence of germ cells, AAV sequences could remain present for prolonged periods, up to 10 weeks[50]. Importantly, clearance of vector from the semen was found to be dose- and time dependent, but serotype-independent[50]. Biodistribution and shedding of AAV2 vectors through other excreta were also shown to be dependent on the route of administration[51].

Retrovirus and lentivirus

Biodistribution of retrovirus and lentiviral vectors *in vivo* is strongly dependent on the type of envelope used. VSV-G is the most commonly used envelope to pseudotype lentiviral vectors and allows transduction of a broad range of cells. There is little data available on shedding of retro- and lentiviral vectors.

Vaccinia virus

Vaccinia is a naturally attenuated strain and is considered a minor human pathogen. Shedding may occur during administration and from skin lesions or body fluids for up to 7-10 days and transmission has been observed to occur in 7.4 per 100.000 primary vaccines after close personal contact[52], as described in detail in Appendix C. As the virus is known to cause severe disease and encephalitis in immune deficient patients, patients with structural brain malformations and young children, this virus should not be used in these patient populations or in patients treated with immune suppressive agents. In addition, since the risk for shedding is considerable, family and close contacts of the patient should be screened for these risk factors as well.

Measles virus

In two recent phase I clinical trials performed by Galanis and Dispenzieri the safety of Measles vectors carrying the CEA transgene and the NIS transgene, respectively, were tested[53]. In the former study 22 patients with advanced ovarian cancer received increasing doses of MV-CEA through ip delivery. Patients did not receive immune modulation and all had pre-existing anti-measles antibodies at enrollment. Fourteen patients displayed dose-dependent stable disease without signs of toxicity. No shedding was measured in any of the patients. In the latter study, 12 multiple myeloma patients were enrolled and treated with MV-NIS through intravenous injection at four different dose levels. All patients had received intensive anti-cancer chemotherapy resulting in profound immune suppression and no detectable preexisting anti-Measles antibodies were detectable. In contrast to the

study of MV in non-immune suppressed ovarian cancer patients, shedding of MV vector could be detected in both blood and throat swab samples (Federspiel, ESGCT 2009). Further trials with MV as an oncolytic vector for the treatment of multiple myeloma patients are currently designed and here Cyclophosphamide will be used as the chemotherapeutic agent of choice due to its demonstrated anti-multiple myeloma-activity as well as its anti-proliferative effects on immune cells.

2.5 Translation of data from animal models to the clinic

2.5.1 Gene therapy animal models using immune modulation

It is unclear how well data from different animal models will predict the behavior of vectors and immune modulation in a clinical setting and, in retrospect, in many cases the clinical relevance of animal models is less than anticipated. In the first place this is, obviously, related to the host-vector specific issues, such as the presence of specific viral receptors and co-receptors on target cells, which determine the species-specificity, organ specificity and infectivity of the viral vectors and therefore biodistribution, persistence and shedding of the viral vectors. For example, a human-derived viral vector may produce different results in an animal and *vice versa* an animal-derived (pseudotyped) viral vector may result in an unpredictable response in human beings. An important example of these species-specific effects is the difference in efficiency of transduction of mouse and non-human primates, with the AAV8 vector being approximately 2 log more efficacious in mice than in monkeys[54]. Another example is the detection of AAV vector sequences in semen of a vector recipient[49], an unforeseen, unwanted effect, not anticipated by previously studied animal models, but later confirmed in a rabbit model[50]. Here it was shown that although AAV vector sequences could be detected in semen, these were found in the seminal fluid, rather than in the sperm cells[50]. In the second place, this is the direct result of immune system and immune modulation-related issues, such as 1) differences in responses to certain immune suppressive agents, eg species-specificity of antibodies, or distinct expression profiles of specific receptors, eg glucocorticoids[55]; 2) differences in immune suppressive treatment protocols; 3) species-related differences in immune responses due to presence or absence of pre-existing immunity and cross-immunity against (human) virus-derived vectors and transgenes, which may affect humoral and cell-mediated responses, eg up to 80% of the human population possesses neutralizing antibodies to some AAV serotypes; 4) differences in T cell responses to viral capsid antigens[56]; 5) the use of immune compromised animals transplanted with human genetically modified cells. For example, there are significant differences in immune responses against AAV between humans and mice. Although AAV in humans cannot activate TLRs and induce type I IFNS, it does induce a CTL response and clearance of the virus. In contrast, AAV infection in mice can activate TLR9 and induce an IFN type I response, but although mice develop a cytotoxic CD8+ T-cell response, these fail to clear transduced cells[56]. In the third and most important place, this is related to the laboratory animal model intrinsically. Most laboratories initiate preclinical testing of viral vectors in specific pathogen free inbred mouse strains. The rationale behind these models is clear and encompasses the relative predictability and homogeneity of immunological responses, depending on the immunophenotypical characteristics of the inbred strain. These type of animal models however, cannot actually be used to predict the risk for recombination with wild type viruses in humans, nor can they be used to assess the risk of viral transfer to secondary or tertiary recipients. The former could perhaps be tested by pre-immunizing animals against the vector or a similar virus and by infecting animals with wild type viruses and measure biodistribution, recombination between viral strains and shedding in excreta, such as mouse droppings. The latter could be easily tested by transferring viral vectors to one or more animals, and measuring shedding and virus production in both treated animals and littermates not subjected to gene therapy treatment. In addition, some animal models may simply not be permissive for propagation of certain viruses, giving a completely different picture of biodistribution and shedding. Furthermore, acquired coexistent infections in humans can act as inflammatory adjuvants at the time of or shortly after gene transfer. This may enhance the host immune response, but is highly unlikely to occur 'spontaneously' in animal models[57]. In the fourth place, the use of otherwise healthy mice as a model for a specific human disease may result in differences in anticipated immune responses. For example, the pathogenesis of HSV in murine and guinea pig models resembles neither HSV-related acute lethality or reactivation[58]. In addition, patients, in whom the underlying genetic defect results in a null phenotype, may display a robust immune response against the foreign transgene, especially if they have not received previous protein replacement therapy, whereas healthy animals may respond with a mild or negligible

response. Or conversely, patients, who have been receiving prior protein replacement therapy, may have in fact developed a tolerance to the transgenic protein and display a reduced immune response[59].

All together, a wide spectrum of limitations confines the choice of the animal model, and interpretation and translation of these data to outbred species, such as humans, should be approached with due caution. The use of non-human primates or other large animals is often required to assess specific responses, safety and toxicity. Although these models may closely resemble the human situation, these studies too should be cautiously designed, to allow optimal translation to the human situation and unforeseen minor differences may result in unpredictable results. For example, differences in dose-responses to glucocorticoids between mice and humans can be attributed to differences in the binding of synthetic glucocorticoids[60] and therefore the use of non-human primates as a model of human innate immune responses after gene transfer is often preferred[61]. However, comparison of immune modulation by glucocorticoids may be less optimal in New World monkeys, due to their glucocorticoid resistance and high levels of circulating cortisol[62]. Another more relevant example is the difference observed between dose-immune response in preclinical tests in mice and monkeys and the Phase I gene therapy trial for ornithine transcarbamylase (OTCD)[63]. The trial was developed as a Phase I dose escalation study using a third-generation adenoviral vector with safety as the primary endpoint. Previous safety tests in rhesus macaques and mice showed a substantially improved toxicity profile with the third-generation vector in comparison with first-generation vector[64]. Furthermore, to assure safety, for the clinical trial the maximum dose of the third-generation vector was chosen to be 17-fold lower than the dose of first-generation vector that showed severe toxicity, including severe liver damage and clotting disorder, in macaques. Simulation of the clinical trial in baboons revealed only minor and transient laboratory abnormalities at the highest tested vector dose[65]. However, during the clinical trial, 17 year old Jesse Gelsinger, who was administered the highest dose of the third-generation vector, experienced an unexpected and dramatic response with systemic inflammation and multi-organ failure, resulting in death[57]. Nevertheless, in studying the effects of immune modulation on gene therapy, under controlled circumstances, NHP models can be advantageous, as there is considerable experience with the use, safety and toxicity profiles of immune suppressive drugs in monkeys; and as drugs developed for humans are often active in NHP, due to the high degree of conservation of protein domain sequences between primates, which is not the case with other mammals.

In conclusion, even if an appropriate animal model is selected for vector testing, differences in dose, mode and route of delivery, eg systemic versus directly into a particular tissue (intramuscular), organ (intrahepatic) or in the vasculature of an organ (hepatic artery), may still affect the subsequent immune response and result in unpredicted consequences in humans. It is therefore of important that new animal models and different readout systems are developed to study specific safety risks.

2.5.2 The use of immune deficient preclinical animal models

The same safety concerns that could be raised when using immune modulation during gene therapy are valid when using immune deficient preclinical animal models, such as nude, athymic or SCID mice. The use of immune deficient animal models is commonplace in xenograft tumor models. Several oncolytic viruses and viral vectors were tested in different xenograft models. The most commonly tested viral vector was replication competent oncolytic Ad for the treatment of glioma. Examples for the use of replication deficient and replication competent viruses in immune deficient animals are given in Table IID and Table IIE, respectively. An interesting study was done to compare the effects of different strains of immune deficient mice on HSV-1 viral replication in tumor tissue[66]. In the immune competent mouse model of oral cancer, HSV-1 produced only limited inhibition of tumor growth and loss of the virus coincided with tumor regrowth. This was likely the result of innate immunity, in particular complement factors, but also humoral factors, inhibiting HSV-1 replication, as previously observed in rats[67,68]. Although the immune deficient mouse strains, ie *nod/scid*, *nu/nu*, *scid/scid* and *scid/beige*, differed with respect to immune function (T, B, NK cells, phagocytic cells) and levels of complement factors, the recovery of virus from infected tumors of each strain of mice did not show important differences from what was seen in the normal immune competent C3H mice with virus disappearing rapidly from the tumors[66]. Although it is likely that oncolytic virus replication and biodistribution in immune deficient animal models is distinct from in immune competent animals, the extent of this difference is dependent on the type of immune deficiency, the type of virus and the type of malignancy. There are little or no data available on the effects of biodistribution and/or shedding in these animals.

2.5.3 Conclusions from preclinical animal models

Adenoviral vectors

Adenoviruses induce rapid innate immune responses due to viral binding to complement, TLR9, erythrocytes, platelets and blood clotting factors (fX) and the adult population has a high level of pre-existing immunity exists against a variety of adenovirus serotypes. The route of delivery contributes to the velocity and type of immune response as does the expression of foreign viral genes, which may even result in direct toxicity. The first generation vectors are more immunogenic than the second generation and the helper-dependent Ad, but even the latter are able to induce a durable immune response due to the presence of immunogenic capsid surface proteins. Whereas the induction of neutralizing antibody formation prevents readministration, clearance of the viral vector from the body is mediated by cytotoxic T-cells. Current methods to prevent or limit immune responses include the use of different (non-human) serotypes, structural modifications or PEGylation. However, all these methods may equally affect the tropism and efficiency of the vector and therefore immune modulation, may be an useful alternative.

The following immune modulatory agents or combinations thereof were used shortly before or during treatment with an adenoviral vector, in most cases Ad2 or Ad5 (see Table II): blockage of co-stimulation (6 studies), anti-T and/or B-cell antibodies (4 studies), cyclosporin A (CsA, 3 studies), cyclophosphamide (CY, 3 studies) and corticosteroids (2 studies). Treatment with corticosteroids resulted in an increased level of vector copy numbers in the liver after intravenous delivery of the vector, but had no effect on the level of transgene expression; treatment with human CTLA4Ig, resulted in the inhibition of formation of neutralizing antibodies and anti-Ad CD4+ and CD8+ T-cells and in a moderately prolonged transgene expression; cyclosporin A treatment had no effect on the formation of neutralizing antibodies, but did show in some cases prolonged transgene expression; cyclophosphamide inhibited activation of CD4+ and CD8+ T-cells, prolonged transgene expression and prevented the formation of neutralizing antibodies. No data are available on biodistribution/persistence and shedding/transmission. No implicit remarks were made on the effect of immune modulation on the number of level of infections in animals during or after treatment. *In vivo*, recombination with a wild type Ad may result in a replication competent virus resulting in prolonged viral replication and dissemination, with the risks for such an event decreasing from first generation, to second generation and helper-dependent Ad. However, it appears that the risks of recombination with wild-type adenoviruses after or during immune modulation are not considerably larger than in gene therapy studies without immune modulation. Furthermore, the risk appears to be more related to the type of vector (first generation, second generation, helper-dependent) and the production procedure (presence of replication competent Ad in the vector batch).

Adeno-associated viral vectors

Early infections with the non-pathogenic adeno-associated viruses results in a low level of pre-existing immunity in the community. Although the AAVs lack pathogeno-associated molecular patterns and are inable to activate toll-like receptors, they still display some level of immunogenicity due to activation of pDCs, cross-presentation of antigens, and macrophage activation by complement C3. The intensity of the immune response is dictated by the route of administration, the level of immune activation (eg as the result of a co-infection with another pathogen), the response against the helper virus (Ad, Herpes, HPV) and reactions against transgenes and transgene products. Clearance and prevention of readministration is mediated by the cytotoxic T-cell response and neutralizing antibodies. Methods to prevent or limit the immune responses include the use of alternative serotypes, pseudopackaging and selection of immune-escape mutants.

The following immune modulatory agents were used in animal studies in combination with AAV-based vectors (AAV-1, AAV2, AAV-6 and AAV-8): cyclophosphamide (5 studies), blockage of co-stimulation with anti-CD40L or CTLA4Ig (2 studies), and combinations of a calcineurin inhibitor, such as CsA or FK506 (Tacrolimus), and Mycophenolate mofetil (MMF) with an mTOR inhibitor (sirolimus) and/or an anti-T or anti-B-cell antibody (rituximab, daclizumab, ATG) in 5 studies (Table II). Treatment with CY generally prevented the formation of neutralizing antibodies against the vector and the transgenic protein, when administered before the gene therapy vector, but when it was used 2 weeks after gene therapy; blockage of co-stimulation resulted in decreased neutralizing antibody formation, prolonged transgene expression, and increased transduction efficiency; the various combination treatment protocols showed some variable effects on prevention of neutralizing antibodies and induction of tolerance to the transgene (but not the vector), but the addition of daclizumab and ATG resulted in unwanted effects, such as loss of transgene expression

due to inhibition of regulatory T-cells and lymphopenia, respectively. Specific data on changes in biodistribution/persistence and shedding/transmission as a result of the immune suppression are not currently available. However, the risks of immune modulation on the environment due to shedding and transmission of AAV-vectors appears to be negligible, due to the fact that 1) the AAV vectors are replication deficient and require several mutations, as well as the presence of a helper virus, to spread, 2) AAV is not associated with any disease, and 3) the presence of (inducible) immunity in the healthy population.

Retroviral and lentiviral vectors

Rapid capsid turnover prevents effective recognition of immunogenic epitopes of lentiviral vectors by activated effector T-cells and immune responses against LV occur almost exclusively in presence of an antigenic transgene[69]. However, immune responses may occur against the LV virion (p17 and p24) itself and envelope proteins. Both retroviruses (MLV-based) and lentiviruses (HIV-based) can induce a moderate innate immune response by APCs (DCs) and a T-cell response. The first generation lentiviral vectors induced moderate immune responses against viral proteins, whereas second and third generation lentiviral vectors, which contain no viral proteins, pseudotyped with VSV-G can induce the adaptive immune reponse due to increased transduction of APCs. Intravenous administration of retroviral vectors in combination with blockage of co-stimulation with CTLA4Ig with or without anti-CD40L was tested in 3 studies (Table II). In general, this resulted in prevention of anti-transgene cytotoxic T-cells, neutralizing antibodies, and induction of tolerance to the transgene. In one study an increase in RV copy numbers was found in the liver, indicating prolonged presence in the circulation. Recombination events with wild type retroviruses resulting in replication competent RV (RCR) are negligible (see also paragraph 2.3), whereas recombination with wild type lentiviruses can only occur in HIV patients, where it may not affect the degree of the disease. It may however lead to prolonged viral replication and dissemination in the patient due to the absence of a complement response or anti-HIV immunity. Due to the rapid capsid turnover, the immune response against LV vectors is predominantly directed against the presence of an antigenic transgene product and gene therapy for HIV is being developed to increase or boost the immune response against HIV antigens. It seems very unlikely that in these cases immune suppressive immune modulation would be required, as the patients are already immune compromised. Due to the rapid capsid turnover, the adaptive immune system is not a major contributor in the clearance of LV particles. Immune modulation directed at suppressing the adaptive immune system may therefore be not a determining factor in the prolonged persistence of the lentiviral vector. Therefore, the risks for the environment are considered unchanged.

Replication-competent vectors

Wild type Herpes Simplex Virus-1 (HSV-1) and HSV-2 are highly immunogenic and induce the full activation of the innate and adaptive immune system, due to rapid complement activation and a TLR2, TLR3 and TLR9-mediated type I IFN response. CD4+ and CD8+ T-cell responses are directed against both structural and non-structural antigens and >50% of the population possesses neutralizing antibody activity against HSV envelope glycoproteins gB, gD, gH-gL. Clearance is mediated by complement, neutralizing antibodies, but most importantly through cytotoxic T-cells. Immune modulation used in preclinical animal models of HSV-based oncolytic gene therapy included dexamethasone, cobra venom factor and CY. In these studies, the primary outcome was the anti-tumor response. Although immune modulation may affect biodistribution and prolong viral replication of HSV, there are currently no corroborating data available. Although the risks for shedding might be increased by immune modulation, the high level of immunity in the community against the HSV provide protection and the presence of the TK gene, allow safety control through treatment with acyclovir and ganciclovir. Other oncolytic viruses such as Reovirus and Vaccinia were tested in combination with CY, but only anti-tumor effects were monitored.

Overview of preclinical animal studies

Table II shows an overview of preclinical studies using a variety of replication deficient (mostly Ad, AAV and scarcely RV) and competent viral vectors (Ad, HSV, Reo, Vaccinia) in combination with different types and combinations of immune modulation. In early childhood most people develop antibodies against the most common adenoviruses and adeno-associated viruses. As a consequence, the use of these types of viral vectors is restricted to less common serotypes, especially in the case of the first generation vectors, which have been shown to induce an early and rapid innate and adaptive immune response in both animals and humans. It is therefore not surprising that particularly the Ad and AAV vectors, and to a lesser extent the HSV and RV-

based vectors are now being tested in combination with different immune suppressing regimens. The immune modulatory agents used in the described preclinical gene therapy studies are typically used for the treatment of cancer or in transplantation patients to prevent organ rejection (see Table III). Extended clinical experience with these types of drugs allows for the development of short-term immune modulatory protocols with sufficient immune suppression to allow delivery of the vectors and tolerance to the transgene without increasing the risk of infections. Immune suppressiva used in gene therapy studies include corticosteroids, alkylating agents such as cyclophosphamide, calcineurin inhibitors such as cyclosporin and FK506, Mycophenolate Mofetil, antibodies against a variety of T- and/or B-cell subsets, and blockage of T and/or B-cell co-stimulation. However, direct translation of these animal studies to the clinics remains complicated due to the use of 1) different animal species, healthy, immune deficient and/or specific disease models; 2) differences in delivery (route of administration) and dose (low, medium, high, single or multiple dosing) of viral vectors; 3) differences in immune responses due to the presence or absence of a functional immune system or pre-existing (neutralizing) immune response against a certain viral vector or transgenic protein; 4) differences in responses towards immune modulatory agents due to species-dependent effects, doses, routes and modes of delivery; and 5) lack of clinically relevant readout systems, such as effects on shedding, biodistribution, vector persistence and recombination after vector delivery.

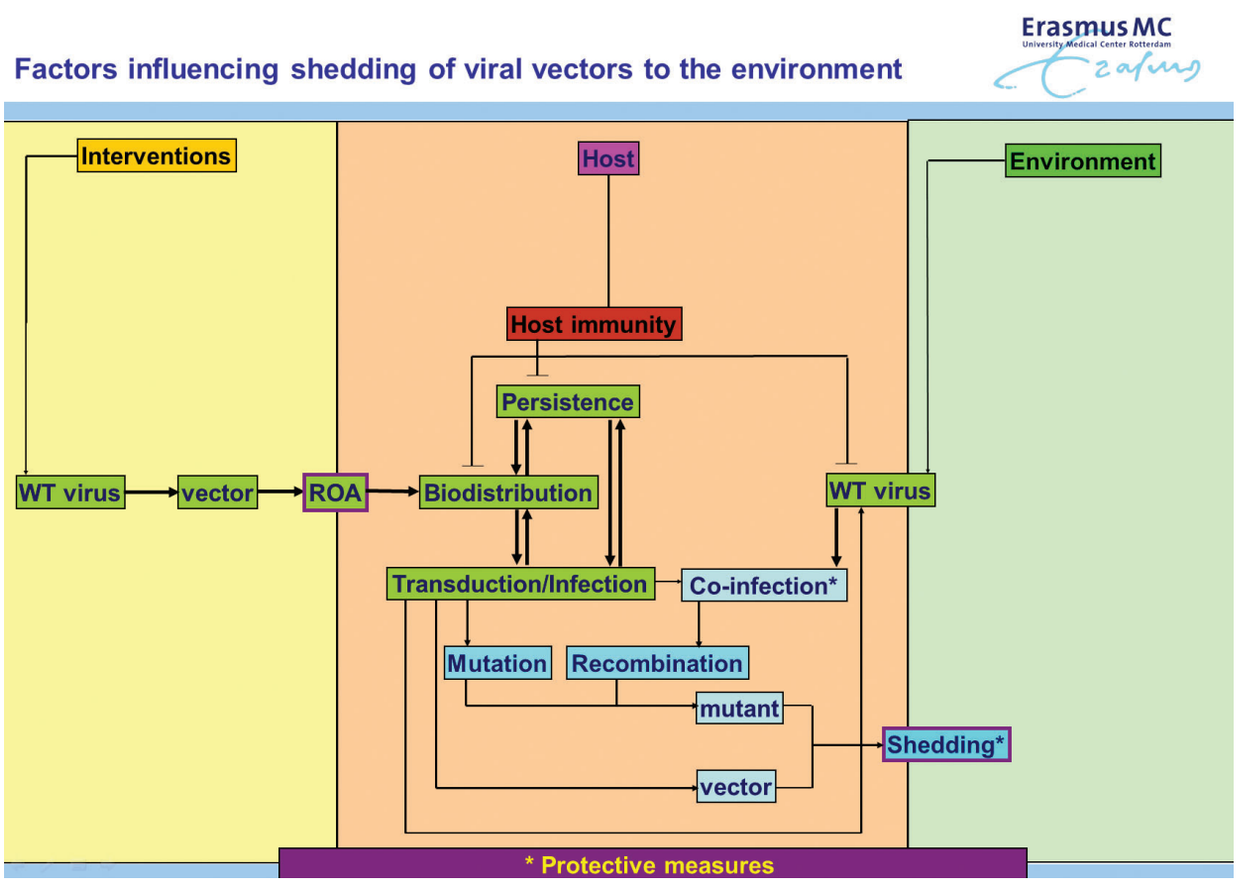
Before starting the project, assessing the risk of immune modulation by studying animal models seemed feasible, but although quite a few different animal models and different types of immune modulation can be found in literature, the aims and readout systems of these studies, ie feasibility of a particular viral vector for the treatment of a specific disease by looking at target tissue transduction and (long-term) transgene expression, are clearly not sufficient to predict changes in viral vector persistence and biodistribution in general. There are currently no data available on the use of immune modulatory agents in clinical gene therapy trials, although some trials for hemophilia[54] and lipoprotein lipase deficiency[70,71] are currently either in preparation or ongoing. Thus, the prediction of risks involved with the use of different viral vectors and immune modulation remains unclear and is largely determined by the characteristics of the viral vectors themselves. In case of a high degree of scientific uncertainty a worst case scenario may be applied in the environmental risk assessment (see also chapter 3).

3 ENVIRONMENTAL RISK ASSESSMENT (ERA)

3.1 Factors and variables determining environmental risk

In order to make a well substantiated environmental risk assessment, it is important to know whether and how shedding and transmission of viral vectors will occur. In addition, the subsequent effect this might have on the environment depends on a number of variables, which are depicted in the simplified scheme 1 (depicted below).

The route of administration (ROA) determines primary biodistribution of the viral vector, and the host immunity determines whether ongoing infection with the viral vector will occur and if secondary biodistribution, or distribution of viral vector after local production in the target organ (eg local, within a specific tissue-type or tumor) will take place. The probability of shedding of viral particles is highest during or shortly after vector delivery to the patient (through direct contact with the inoculation site), but remains present for a certain period of time after the vector has been delivered and persisted in the patient's body. Furthermore, the length of this particular period depends on the capacity of the vector to replicate. In case shedding occurs during or shortly after delivery, this will only result in transfer of unmodified viral vector, with a known tropism, virulence and immunogenicity. In case shedding occurs after persistence of the viral vector in the patient, either shedding of the unmodified viral vector may take place, but also, theoretically, an altered viral vector may be shed. The latter can be divided in two groups: 1) alterations resulting in a reversal to a wild-type virus, as observed in viral vectors with a single modified gene, generating a virus with a known tropism, virulence and immunogenicity or 2) recombination with a related wild-type virus, which may occur due to co-infection with an endemically circulating virus or due to reactivation of a latent infection and may result in a virus with unknown tropism, virulence and immunogenicity. Reversal to the wild type virus is for the environmental risk assessment of less importance, as in this situation the risks are not increased in comparison to the normal situation. In practice, the chances of recombination, depend on the type of vector (replication competent vs deficient), the type of virus (RNA/DNA),

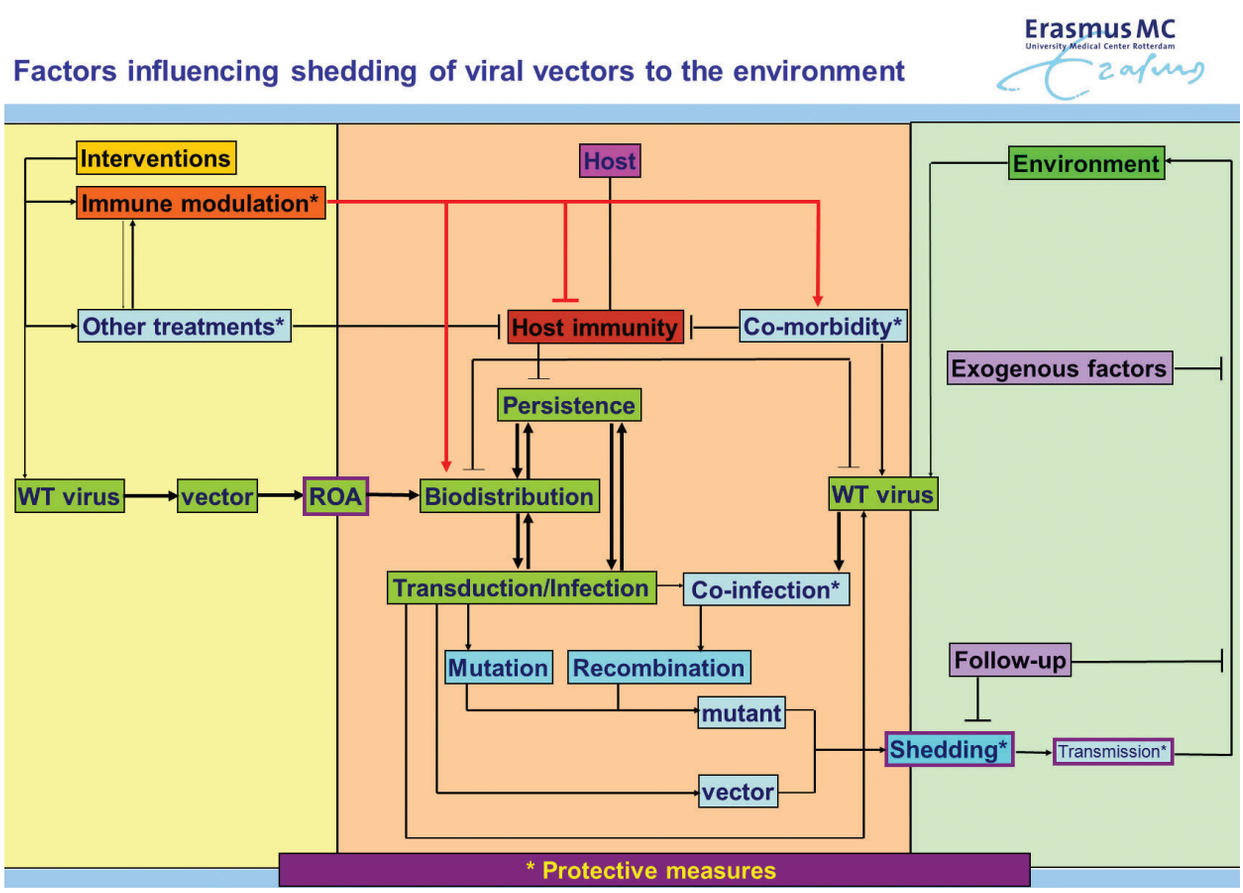


Scheme 1 Factors influencing shedding of viral vectors to the environment. After administration of modified wild type (WT) virus, the route of administration (ROA) determines biodistribution. The type of vector used, ie replication competent or deficient, determines further infection of cells and persistence of the vector in the host. Loss of transgenes or complementation of viral functions may result in reversal to WT virus, other mutations can result in a new potentially dangerous virus. Shedding to the environment may take place during administration to the patient, but also after persistence in the patient.

the time needed to complete a single viral life cycle, and whether the virus is lytic or not. Good compliance with protective measures during vector administration, hospitalization and after discharge from the hospital as well as education of laboratory/health care personnel and house-hold members can prevent transmission of (modified) vector strains. Alternatively, increased safety can be provided by vaccination of bystanders (personnel) at risk for example when replication competent Vaccinia virus is used or certain types of Adenovirus, eg Ad5[72]. Careful follow-up of patients, their house-hold members and health care personnel is required as long as infectious viral particles can be detected in patient excreta.

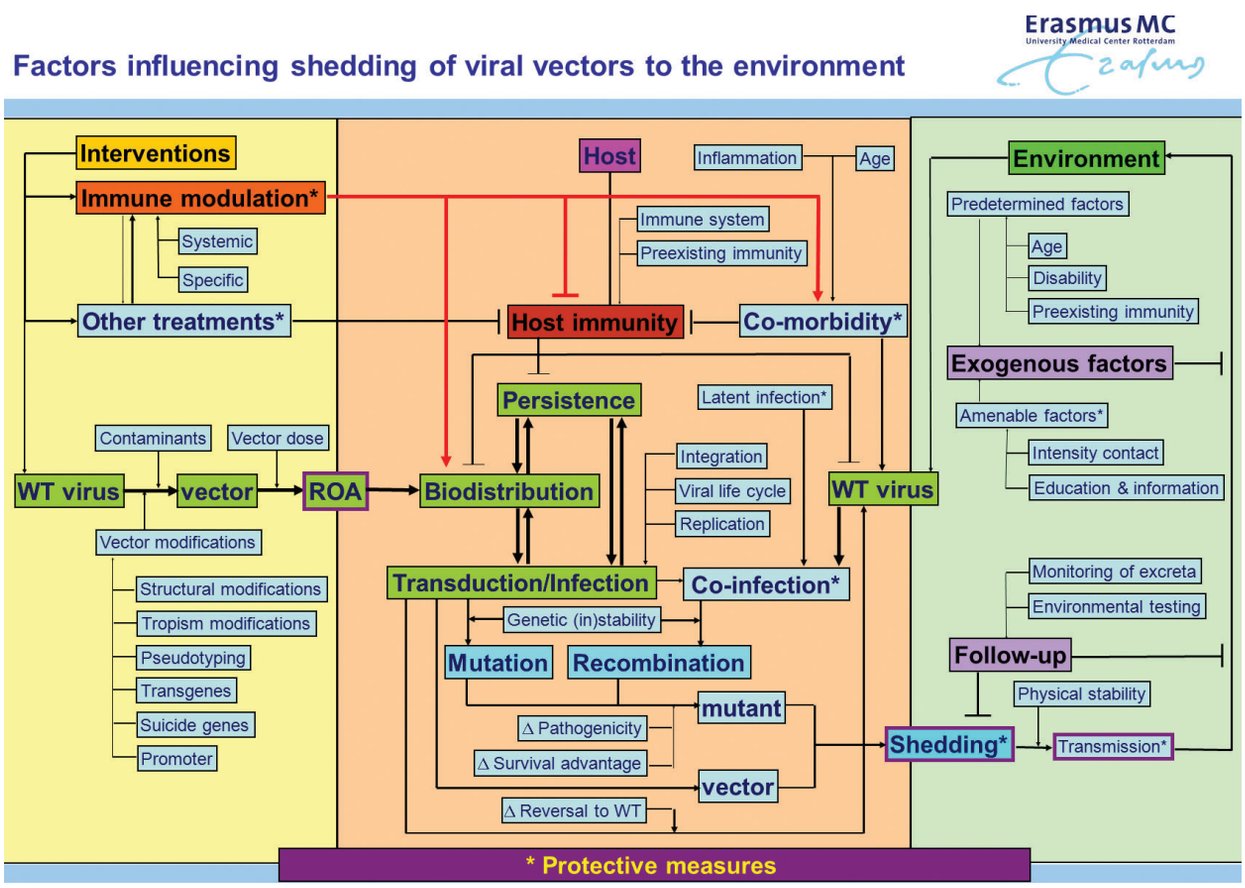
Immune modulation can affect these variables at several levels by interfering with the host immunity (red arrows). This may result in increased viral vector persistence and possible changes in biodistribution and continuous infection. Suppression of the host immune system may also result in an increased sensitivity to certain pathogens, in particularly herpes viruses and adenoviruses, and lead to primary infection or reactivation of latent viruses. These two effects of immune modulation could result in co-infection and an increased risk for recombination (scheme 2). Co-morbidity, which might render the patient relatively susceptible to infection, or diseases which affect the host immune system and other treatments, which affect the immune system directly or may interfere with immune modulatory agents, can therefore by default also affect the risk for co-infection and recombination.

Other elements that may interfere, include specific vector modifications (**Δ**: deletions or additions) determining tissue tropism, immunogenicity, replicative capacity, and function of the viral vector; the type and duration of immune modulation used, the presence or absence of preexisting immunity (Scheme 3). The effects on the environment depend strongly on whether the shed virus has a survival advantage, the pathogenicity, and physico-chemical properties determining stability of the virus outside of the body. Factors affecting the risks for transmission include the intensity of the contact, pre-existing immunity in the general population, education and information of health care personnel and household members, as well as the physical condition of the latter. Monitoring of shedding and environmental testing is imperative.



Scheme 2 Influence of immune modulation and co-infection: opportunities to minimize risk. Immune modulation can affect biodistribution and persistence of viral vectors, by inhibiting the immune system, but can at the same time also increase the risk for co-infection with a wild type virus. Other treatments or comorbidity may interfere with immune modulatory agents or directly affect the immune system by itself. These factors all play a role in the increased risk for recombination with WT viruses and possibly with shedding.

In case of recombination, the potential environmental risks of the newly formed mutant virus are dependent on its capacity to survive and procreate, but its direct clinical effects are dependent on its virulence. Under normal circumstances, a virus needs to clear a number of hurdles to be able to cause disease, including invasion of the host; replication in susceptible cells at the site of inoculation; resistance of local defense mechanisms; spread from the site of inoculation; replication in target tissue; and eventually exit from the host. Upon direct injection of conditionally replicating viral vectors into the blood stream or in a specific tissue, not only many of these hurdles are bypassed, but also the inoculation dose is multiple folds higher than with natural infections. Replication and possible recombination can only occur in susceptible cells, depending on the (changed) tropism of the viral vector and are more likely to occur when the virus is non-lytic and has a slow life cycle. In patients, in whom local defense mechanisms may be decreased due to illness and/or treatment-related immune suppression a certain level of replication of a mutant virus may be allowed. In contrast, in otherwise healthy bystanders, such as relatives and health personnel, with a functional immune system, much depends on how well the newly formed virus is able to enter a new host and how/whether the host immune defenses will respond appropriately to the newly formed virus. With age the level of immunity against the most common viruses increases, such as adenoviruses (personal communication with Marco Schilham). This implicates that younger subjects are not only more likely to allow virus propagation for a longer period of time, it also indicates that younger bystanders are more likely to be susceptible to new virus infections. The virulence of this virus in a healthy population is largely dependent on the tropism of the new virus and thus depends on the presence of appropriate receptors on the cell surface of susceptible cells. In a suboptimal environment only partial replication may take place, thus resulting in an incomplete form of the virus with either severely diminished or no capacity to infect other cells. It is conceivable that the cellular environment in which the mutant virus is formed may therefore not be able to sustain a continued infection. Moreover, whether the life cycle of the recombinant virus will be completed (ie. is the virus complete and can it independently replicate and can it be functionally packaged) and how and when the



Scheme 3 Total of factors and variables determining the environmental risk assessment. Many other factors may influence the risks of recombination and shedding as well, such as specific vector modifications, the type and stability of the virus, the transgene, possible survival advantage, preexisting immunity, etc. However, transmission can be prevented in most cases by using preventive physical barriers.

mutant virus will be eliminated from the circulation are determinants of the environmental risk. Examples are the formation of incomplete retrovirus variants or the recombinantion of wild type Ad with Hd-Ad, resulting in genomes too large to package.

3.2 Background information for the ERA

When assessing the effects of immune modulation on viral persistence, risk for recombination and shedding/transmission, some issues need to be addressed. Firstly, immunogenicity of different vectors requires different levels of immune suppression, whereas the mechanisms through which the vectors induce immune responses dictate the choice for a particular immune suppressive agent. Although oncolytic Vaccinia and HSV vectors are attenuated, they possess their complete immune evasion machinery. Thus, despite the fact that many people are immunized against Vaccinia and that more than half of the population has antibodies against HSV, this does not prevent effective use of these vectors or affect the immune response against these vectors or the transgenes. However, pre-existing neutralizing antibodies against adenovirus severely affect transduction efficiency of the replication-defective vectors, but are less important for the conditionally-replicating vector ONYX-015[73]. ONYX-015 had a deletion in E1B-55 kDa and E3B, but still possesses some of the remaining immune modulatory E3 genes[74]. ONYX-015 induces a rapid humoral anti-Ad immune response, but safety or efficacy of treatment with ONYX-015 in cancer patients with and without pre-existing antibodies was found to be comparable[73]. Secondly, the environmental risk is dictated by many factors that are not influenced by immune modulation, such as age, type of vector (conditionally replicating/replication defective), transgene (toxic, antibiotic resistance), etc.

The report "Environmental risk assessment for replication competent viral vectors in gene therapy trials" as published in 2008 by the RIVM[75] contains a list of points to consider when applying for a licence to use replication competent viral vectors in humans. The report can be downloaded at http://www.rivm.nl/bibliotheek/rapporten/601850001.pdf. The immune modulation environmental risk assessment questionnaire is based on this existing list. Part A and B discuss general, treatment and viral vector related issues and have been modified where necessary to be able to assess risks and interactions with immune modulation treatment. Part C has been added for this report and discusses issues related to immune modulation. A short overview of this new ERA is given at the next page and all issues are separately discussed in detail below. To clearify why certain topics are specifically important for the assessment of risks involved with immune modulation, small blocks of background information are provided. This will help the user to understand the reasons for these particular questions and may be helpful for future applications. In the ERA, we chose to use the Alipogene Tiparvovec (AMT-011) AAV1-LPL^{S447X} vector as an example. The rationale behind this was the recent initiation of phase I and phase II/III trial with this vector by AMT in Canada[70,71], making this the most relevant example with respect to clinical applications in the near future. Another relevant example could have been the currently recruiting phase I trial for Hemophilia B with AAV2-hfIX[54]. However, the checklist can be used for all types of vectors, not only AAV, and is also relevant for other types of immune modulation, mediated for example through the use of stealth technology.

ERA TEMPLATE FOR IMMUNE MODULATION GENE THERAPY

Part A:
General and treatment-related issues

Step 1: Describe the patient population
1a. How many patients are included in the clinical trial
1b. What is the age range of the patient population
1c. Describe the disease (genetic, cancer, etc)
1d. Does the disease affect the immune-responses of the patient
1e. What are specific in/exclusion criteria
1f. What are the primary and/or secondary endpoints

Step 2: Viral vector administration
2a. What is the route of administration (ROA)
2b. What is the way of administration
2c. What is the dose and timing of administration
2d. Is the target organ an immune-privileged site

Step 3: Information about other treatments applied to the patient population
2a. Treatments directly affecting the immune system
2b. Treatments affecting pharmacokinetics of viral vectors
2c. Treatments affecting pharmacokinetics of immune modulatory agents

Step 4: Information about the patient's environment
4a. Hospitalization-related information
4b. Housing-related information
4c. Out-house activities
4d. Animal contacts (occupational or recreational)

Step 5: Information about protective measures used to prevent transmission
5a. To healthcare personnel
5b. To household members
5c. How long will these measures be continued
5d. How is compliance with the measures assessed

Part B:
Vector-related issues

Step 1: Information about the wild type virus
1a. Which wild type virus is used as backbone for the vector
1b. Infectivity of non-replicating cells
1c. Integration into host genome
1d. Virulence and pathogenicity
1e. Host-range (human/animal, broad/restricted)
1f. Tissue tropism
1g. Biodistribution
1h. Persistence
1i. Cell lysis and lateral spreading
1j. Innate immune response
1k. Viral clearance
1l. Horizontal transmission
1m. Vertical transmission
1n. Genetic stability
1o. Availability of anti-viral treatment
1p. Physical and chemical stability
1q. Immune evasiveness

Step 2: Information about the viral vector
2a. Is the vector replication competent
2b. Can the vector infect non-replicating cells
2c. Can the vector integrate into the host genome
2d. Information about deletions of viral sequences
2e. Information about inserted transgenes or sequences
2f. Replication of the viral vector in normal cells
2g. Immune evasiveness of the viral vector
2h. Availability of preclinical models
2i. Information about tropism, targeting and expression
2j. Information about biodistribution after injection
2k. Information about persistence after *in vivo* administration
2l. Information about mutation rates and recombination *in vivo*
2m. Information about possible toxicity
2n. Environmental shedding
2o. Horizontal transmission
2p. Safety back-up

Step 3: Information about production of the vector
3a. Which producer cell lines are used
3b. Which viral functions are provided by the cell lines
3c. Which quality control measures are used
3d. Which criteria are used to reject a batch

Part C:
Immunity-related issues

Step 1: Information about host immunity
1a. Does the patient have a functional immune system
1b. Does the patient have a condition affecting immunity
1c. Does the patient have pre-existing or cross-reacting immunity
1d. Is the patient a carrier of a related virus

Step 2: Information about immune modulation
2a. What is the type of immune modulation(s) used
2b. What is the dose and duration of the treatment(s)
2c. What is the level and duration of immune suppression
2d. Explain the choice of immune modulation

Step 3: Effect of immune modulation on the risk of infection
3a. Increased risk for primary infection
3b. Increased risk for reactivation of latent infection

Step 4: Information about other treatments
4a. Which other treatments are used, at what dose and duration
4b. Do these treatments affect the immune modulatory agents
4c. Do these treatments affect the patient's immune system
4d. Do these treatments influence vector kinetics

Step 5: Effect immune modulation on vector distribution and persistence
5a. What are the relevant animal/clinical studies
5b. Does immune modulation after vector biodistribution
5c. How are biodistribution and persistence measured
5d. How long is biodistribution/persistence measured

Step 6: Effect of immune modulation on recombination
6a. What are the relevant studies
6b. How are recombination or reassortment affected

Step 7: Effect of immune modulation on risk for shedding and transmission
7a. What are the relevant (pre)clinical studies?

PART A: GENERAL AND TREATMENT-RELATED ISSUES

Step 1: Describe the patient population

1a. How many patients are included in the clinical trial?

1b. What is the age range of the patient population?

Background

In the first few years of life, the immune system is still naïve and highly sensitive to new infections. In addition, the high virus load in the excreta of these children increases the risk of infection of members of the same household (siblings) as well as their peers. Also elderly are particularly susceptible to infections, which may affect their responses towards a viral vector (see below). Of the viruses used as vectors, particular interest should be given to HSV and VACV. Wild-type HSV-1 infection in pre-immune children (neonates) and immune incompetent patients can result in HSV encephalitis[76]. Although HSV-vector induced encephalitis has never been reported in humans, in animal models it was shown that high doses of first generation oncolytic HSV vectors could induce significant morbidity and mortality[77]. Also sensitivity to VACV is particularly high in preimmune children below the age of 1 year[78-80] and immunocompromised patients and infection can lead to progressive vaccinia, postvaccinial central nervous system disease, and eczema vaccinatum. Before the age of 5 children are prone to Ad infections and develop a long-lasting cross-reacting immunity. Although the youngest, pre-immune children and the elderly are at increased risk for infections, in many trials they are the two target groups for gene therapy: the youngest to treat genetic diseases, the eldest to treat neoplastic growth. Age is an important risk factor for patient-related infectious risks.

Possible effects immune modulation

Age can affect the choice, dose and regimen of immune modulation, with for children the preference for specific immune modulatory agents over systemic and cytotoxic immune suppressives and for elderly the possibility of interaction with other medication. Increased risks for infections, observed in certain age groups, may be aggrevated due to use of immune modulation and allow for an increased risk of co-infection and recombination.

1c. Describe the disease (genetic, cancer, etc)

1d. Does the disease itself affect the immune responses of the patient?

1e. What are in/exclusion criteria

1f. What are the primary and/or secondary endpoints of the study?

Background

The route of administration strongly affects the biodistribution and evoked host immune reponse. *Ex vivo* viral gene transduction is not likely to induce a host response against the vector itself, but the route of administration of transduced cells may nevertheless affect the immune response against the transgene. *In vivo* administration of viral vectors is bound to induce an immune response, but route of administration may affect the intensity and velocity of the induced immune response. Immune modulation can be used to decrease the immune host response against both the viral vector and/or the transgene. Immune modulation in combination with *ex vivo* gene transfer could be used to induce tolerance for the therapeutic gene.

Step 2: How is the viral vector administered?

2a. What is the route of administration (ROA)? (systemic: sc, ip, iv, ia; or local: intratumoral, intralesional, intrathecal, intrapulmonary.)

2b. What is the way of administration? (injection, inhaler/spray, other)

2c. What is the dose (pfu, vp, ip) and timing of administration (single dose, multiple doses, time in between treatments)?

Background

A single dose may not require the use of immune modulatory agents, whereas multiple doses for optimal effect may require accessory immune suppressive treatment, even if injected in immunoprivileged sites[81]. A large viral load is a potent stimulus for pattern recognition receptor (PRR) signaling, and receptors such as TLR7 are activated by exposure to engineered viral genomes[82]. A large viral load can also satisfy the system and induce significant toxicity[83], eg binding of Ad vector to platelets after intravenous administration can result in induction of thrombocytopenia. Multiple dosing or high dose treatments to achieve sufficient therapeutic effect may require multiple doses of/protracted immune modulation therapy, with increased risks for infections, recombination, etc.

2d. Is the target organ an immune-privileged site or not

Background

Certain compartments of the body, such as the brain[84], eye, placenta and testes[85], are either naturally tolerant or experience attenuated and delayed immunologic responses to newly introduced foreign antigens[86]. These anatomical sites are said to be immune privileged and a certain level of tolerance is maintained through multiple mechanisms, including the blood-brain barrier[87], the blood-testis barrier and limited blood supply to the cornea or lack of MHC class I and II expression. This immune privilege is however not absolute but relative in comparison to other tissues and increasing doses of immunogenic antigens, such as viral vectors, will result in an increased inflammatory response and elimination of the foreign gene[83]. Direct injection of increasing doses of Ad-LacZ from 10^6 up to 10^8 infectious units into the mouse striatum resulted in increased transgene expression, reaching a plateau at vector doses of 10^8 i.u. and minimal cytotoxicity, but doses exceeding this level resulted in cytotoxicity due to upregulation of IFN-type 1 regulated genes and chemokines[88] leading to acute inflammatory-induced cell death and loss of transgene expression[83]. Similarly, doses of 10^2 to 10^6 transducing units of SIN-LV-GFP in the brain resulted only in a minimal increase in inflammatory markers[69]. Systemic immunization of animals with Ad[89], but not LV[69], or with the transgene can result in an CD4+ and CD8+ T-cell[89] and B-cell[90] mediated adaptive immune responses and loss of transgene expression in the immunoprivileged site. In addition, a preexisting immune response against Ad is insufficient to block vector transduction and transgene expression from FG or HDAd vectors injected in brain of immunized animals[91-94]. Transient, innate immune responses may occur when AAV vectors are injected into the brain parenchyma[95] and, as with other viral vectors, are likely dose-dependent, with low doses in the order of 2-4x10^8 particles inducing little or no detectable immune responses[81,96], and high doses of 4x10^{10} particles or multiple doses inducing significant transient intrastriatal inflammation[95]. Maintenance of immune privilege is not an easy task, and is influenced by age[84]. Immune modulation is indicated if a preexisting immune response exists against either the vector or the transgene and affects the transduction efficiency. For replication deficient vectors short-term immune modulation could suffice, as viral capsid proteins are only presented shortly during initial processing of the virions; for replication competent vectors, induction of tolerance or prolonged immune suppression is required. In immune privileged sites, the use of immune modulation is most likely only required upon multiple injections, or when high vector doses are needed to obtain a clinical response.

Possible effects of immune modulation

Immune modulation is likely to affect biodistribution and persistence of replication deficient or replication competent vectors when administered systemically, but upon local injection in an immune privileged site, such as the brain, short-term immune modulation may allow optimal transduction without negative effects on biodistribution and shedding, thus shifting the balance towards increased efficiency. Nevertheless, it should be noted that small molecules may diffuse through the brain and may not only activate local innate immune responses, but also an adaptive immune response, leading to loss of efficacy.

Step 3: Information about other treatments applied to the patient population

Required information

Information is required concerning all treatments, other than gene therapy, that are provided shortly before and during the gene therapy procedure and recovery period, such as chemo- or radiotherapy, growth factors, (re)vaccination, angiogenesis inhibitors, other agents affecting the immune system or biodistribution and persistence of the viral vector, etc.

3a. Treatments directly affecting the immune system
3b. Treatments affecting pharmacokinetics of viral vectors
3c. Treatments affecting pharmacokinetics of immune modulatory agents
3d. Other

Step 4: Information about the patient's environment

Possible effects immune modulation

The environment itself can influence the risk of shedding to the environment by affecting the contact-zone, the intensity and duration of contact, etc.

4a. Hospitalization-related information
4b. Housing-related information, including household information, nursing-home
4c. Out-house activities, such as profession, military, kindergarten/schools, sportsclubs, etc

4d. Animal contacts (occupational or recreational)

Possible effects immune modulation
The total of effects of immune modulation determines the risk for recombination or shedding, but whether transmission will take place between a patient and its environment is not directly dependent on the immune modulation, but on the intensity of the contact and the compliance of the patient and its environment to apply the appropriate precautionary measures.

Step 5: Which protective measures are utilized to prevent transmission?

5a. To healthcare personnel
5b. To household members
5c. How long will these measures be continued, eg before, during and after therapy
5d. How is compliance with the measures assessed

Possible effects immune modulation
Depending on the type and duration of immune modulation used, the usage of protective measures may have to be prolonged.

PART B: VECTOR-RELATED ISSUES

Step 1: Information about the wild type virus

Background
The characteristics of the wt virus determine largely the risks encountered with the vectors and provide information about the mechanisms employed by the vectors to infect, spread, shed and recombine. The characteristics of the wt viruses used as backbones for vectors that are used in combination with immune modulation are summarized in Table IV.

Required information
This section should include all relevant scientific information about the wild type virus and/or modified (lab)strains on which the viral vector is based.

1a. Which wild type virus is used as a backbone for the vector
1b. Infectivity of non-replicating cells (Table IV)
1c. Integration into host genome (Table IV)
1d. Virulence and pathogenicity (Table IV)
1e. Host-range: Human/Animal, Broad/Restricted (Table IV)

Background
Viruses, which occur naturally in non-human species and are non-pathogenic for humans seem a logical choice to use as viral vectors. However, if modified replication-competent vectors become pathogenic due to (a series of) recombination and mutation and encounter an immunological naïve population, there is a risk for a possible epidemic[97]. From a safety perspective, an oncolytic replicating vector should therefore be derived from viruses, which are naturally endemic among the human population. The use of dangerous human pathogens is inadvisable, due to the risk of reversion to wild-type or recombination with wild-type into an even more virulent strain, with a possibly different tropism. Therefore, the best approach to develop a replication-competent vector would be the use of a highly prevalent but only weakly pathogenic human virus, such as Ad. Reversion to a wild-type phenotype would then not result in a serious risk for the patient and upon inadvertent release in the human population, the mutant virus would be encountered by (cross-reacting) neutralizing antibodies, and disease burden would be low. However, the possibility that recombination or mutation could give rise to a more virulent variant of the virus remains an important issue, and the risk is largely determined by the tissue tropism, the structural modifications and the promoter used in the vector, as well as the transgene involved[97]. Modifications restricting the tropism of the vector, in comparison to the wild-type virus, would not pose a serious risk, whereas the choice of the transgene may have important implications (see below).

Possible effects of immune modulation
During early life people develop neutralizing antibodies against the most common adenoviruses and adenoassociated viruses. To prevent an instant immune response, viral vectors can be pseudotyped or alternative, less common or animal serotypes can be used. Immune modulation is only indicated if a significant immune response is anticipated, eg if the dose of the vector is high, if an immune response exists against the transgene, or if multiple doses of vector are administered. Immune modulation may allow for increased persistence and altered bio-distribution of the viral vectors and possible prolonged shedding of viral vectors which are not recognized by the general population. The risk for the general population upon transmission depends on the immunogenicity of the viral vector and possible cross-reactivity, and the function of the transgene (see Table V).

1f. Tissue tropism (Table VI)
1g. Biodistribution

Background
Primary biodistribution is strongly dependend on the route of administration (local/systemic) and whether the target tissue is an immune privileged site or not. Secondary biodistribution depends on the host immunity and whether the vector is replication competent or not.

1h. Persistence
1i. Cell lysis and lateral spreading (Table IV)

Background
The time available for recombination is shorter for viruses that spread by killing and lysing the cells in comparison to viruses that spread through cell-to-cell contact.

1j. Innate immune response (Table IV) and in detail discussed in appendix A.
1k. Viral clearance (Table IV) and in detail discussed in appendix A.

1l. Horizontal transmission: Specific characteristics of wt viruses, affecting transmissibility are summarized in Table VII
1m. Vertical transmission
1n. Genetic stability/Recombination (Table IV)
1o. Availability of anti-viral treatment (Table IV)
1p. Physical and chemical stability (Table IV)
1q. Immune evasiveness (Table IV)

Background
The intensity of the immune response depends largely on virus's capability to hide itself from the immune system, the socalled immune evasion mechanisms. Replication-competent oncolytic vectors often possess all or most of the viral evasion equipment, but in replication defective vectors many of the genes that viruses use to evade the immune response are removed to increase safety and/or to allow the insertions of larger genes. For example, in the helper-dependent Ad vectors, all viral genes have been removed, except the ITRs and the packaging signal and these vectors may cause an even more robust immune response in reaction to the viral vector than to the wild-type virus. Other factors affecting evasiveness include, shielding and variability in antigenic structure. Viral evasion mechanisms are discussed in Appendix A.

Step 2: Information about the viral vector

Required information
All relevant scientific information about the viral vector should be addressed here.

2a. Is the vector replication-competent?

Background
As with live-attenuated vaccines (see Appendix C), replicating viral vectors may raise serious safety concerns with respect to pathogenicity, mutation rate/risk and risk of recombination, risk for spread of a mutated pathogenic vector and risk for germline transmission[98]. If pathogenic mutations occur in the replication competent vectors, this could potentially lead to serious epidemics in susceptible populations, particularly under conditions which favor transmission (see above). Where the first generation replicating vectors had deletions in a single gene, the second generation replicating vectors contain multiple deletions, rendering the viruses safer and with a decreased risk to reverse to a wild-type variant.

Possible effects of immune modulation
Recombination can only occur during the life cycle of the virus. Replication-competent viruses allow for recombination with wild-type viruses during each life cycle. Immune modulation may decrease the host immune response against the viral vector and allow for longer viral persistence and changes in biodistribution. Both may increase the risk for recombination with wild type viruses and risks associated with shedding of the viral vector.

2b. Can the vector infect non-replicating cells?

Background
Some wild type viruses, such as HSV, may remain latent in non-replicating cells, eg in neurons.

Possible effects of immune modulation
Immune suppression may result in reactivation of latent viruses, in particularly herpes viruses and recombination with HSV-based vectors could occur under these circumstances.

2c. Can the vector integrate into the host genome?

Required information
Provide relevant information on the location of the integrations (preferential sites, genes, chromosomes) and possible results thereof.

Background
The two major issues related to viral integration are insertional mutagenesis, which may occur when a virus integrates in a somatic cell, and germline transmission. All dsDNA viruses and retroviruses, which synthesize DNA during replication, have the potential to be oncogenic through insertional mutagenesis.

Adenoviral vectors
After infection of cells Ad rarely integrates into the genome: infection with wild-type Ad of permissive cells leads to lytic infection, but integrations may occur in non-permissive cells (e.g., hamster cells infected with Ad12)[99,100], or under non-permissive circumstances (e.g., specific temperatures with Ad5)[101]. Although transformation of cells after infection with adenoviruses has been observed in culture systems[102], adenoviruses appear not to cause tumors *in vivo* in humans during the natural course of infection. Transformation of mammalian cells can be achieved with DNA plasmids carrying no more than the Ad E1 region[103], and

although for transformation of rodent cells E1A or E1A and E1B-19K suffice, it is much more efficient in presence of the E1B-55K protein[103-105]. In contrast, for the transformation of human cells the full Ad E1 region is required[106,107]. The oncogenic phenotype of Ad serotypes is determined by the E1A[108,109] gene, which modifies the function of key regulatory proteins such as retinoblastoma (Rb) and the chromatin remodeling protein p400, an inducer of the cellular oncoprotein Myc[110,111], but is deleted in most adenoviral vectors. The role of E1B-55K in tumorigenesis is however less clear. It was shown that the protein binds and inhibits p53 and has an anti-apoptotic function[112,113]. The E1-substituted and helper-dependent Ad vectors show integration efficiencies of respectively 10^{-3} to 10^{-5} per cell. The higher integration efficiencies of the helper-dependent vector can be attributed to the lack of viral genomic sequences or to the lack of leaky expression of viral genes, which may inhibit normal cellular machinery, unlike those in the E1-deleted vector. Analysis of host cell chromosomes revealed that most genomes contained extra Ad vector fragments and that although gene expression from the integrated vector was relatively stable, integrated vectors may be subject to further rearrangements and altered gene expression[114]. However, the replication-deficient vectors are deleted for E1, and most of the conditionally replicating adenoviruses, such as ONYX-015, are deleted for E1B-55K. It was suggested that the helper-dependent adenoviral vectors might have a higher risk for insertional mutagenesis, due to the vast amount of genomic stuffer DNA which might facilitate integration through homologous or homology-mediated mechanisms. In contrast, like E1-deleted vectors, integration occurred at randoms sites, mainly by insertion of a monomer with close to no loss of sequences at either vector end[115].

Adeno-associated virus-based vectors

Although the genome of single stranded AAVs can stably integrate into host-cell DNA, the naturally occurring AAVs are not associated with oncogenesis. However, rAAV vectors lose their ability for site-specific integration due to deletion of the rep gene and may integrate randomly[116,117]. Two studies documented insertional mutagenesis in neonatal rodents as a result of integration of rAAV vectors[118-120]. Whether the risks for insertional mutagenesis can be translated to a clinical setting remains to be seen and depends on a number of factors. Thusfar only few reports of tumorigenesis in neonatal rodents have surfaced and none in other animal species, but other factors, such as the vector dose required to transduce sufficient cells[123], the route of delivery and the immune response toward transduced cells[49] may affect the outcome in humans and make it difficult to predict the actual risks. In addition to the risks of tumorigenesis, also the risks for true germline transmission appear low[48,49]. Patients with hemophilia B treated with rAAV-fIX showed short-lived expression of therapeutic fIX levels after injection in muscle[124,125] or direct into the hepatic artery[49], as a result of the induced host immune response. However, after injection into the hepatic artery, for up to 12 weeks vector sequences were detected in semen, even in the lowest dose treatment group and clearance was more quickly by younger than older patients[49]. Fractionation of the semen demonstrated the presence of vector DNA in the seminal fluid and no evidence was found of vector sequences in motile sperm[49]. This is in agreement with data showing that AAV2 does not transduce spermatogonia directly[126,127]. In addition, data from intravenous AAV2 and AAV8 vector transfer to vasectomized rabbits demonstrated that the presence of vector sequences in seminal fluid[50]. Thus, no true germline transmission could be found, but shedding in seminal fluid was found to be transient in all animals and clearance was found to be dose- and time-dependent, but serotype-independent[50]. Therefore, it was recommended that subjects use barrier contraception until the semen becomes negative for vector sequences[49].

Herpes virus-based vectors

EBV has been directly linked to the occurrence of Burkitt's lymphoma, but although there are some reports associating HSV-2 with cervical cancer, evidence of a direct link with HSV is circumstantial and often a second infection with either HPV or Chlamydia is present. HSV generally does not integrate, but exists episomally, making the risks for insertional mutagenesis very low. HHV6, was recently shown to be unique among Herpes viruses in that it efficiently integrates into telomeres of chromosomes during latency, rather than forming episomes and that the integrated viral genome is capable of producing infectious virions[128]. Other DNA viruses such as HPV and the hepatitis viruses HBV and HCV are associated with oncogenesis as well, but as they are not commonly used as gene therapy vectors, the discussion of these viruses is beyond the scope of this project.

Retroviral vectors

Germline infections of ancestors millions of years ago have resulted in the presence of human endogenous retrovirus (HERV) sequences, which make up 8% of

the human genome[129]. Some of the HERV members have undergone repeated outbursts of replication, resulting in more than 60 proviral copies and over 2500 solitary LTRs[129,130], and the proviral elements of HERV-K not only still retain their open reading frames for all viral genes[131], but the various loci of HERV-K elements can code for all structural, regulatory and enzymatic viral proteins[130]. In addition, at least 50% of human-specific HERV-K LTRs serve *in vivo* as active promoters for nearby genes[132]. The four known human exogenous retroviruses are HTLV-1, HTLV-2, HIV-1 and HIV-2. The HTLV-1 Tax protein can cause adult T-cell leukemia due to activation of cellular proliferation in 1-2% of infected patients, often after a latency of up to 50 years[129]. In animals, oncogenic retroviruses, such as MLV, MMTV and FeLV, can transform normal cells by 3 mechanisms, i.e. by picking up cellular oncogenes and subsequent co-infection with a wild type helper virus for replication, by insertional mutagenesis resulting in destruction or disruption of tumor suppressor genes, or by downstream activation of nearby proliferation supporting cellular genes[129].

Retroviral vectors based on MLV display preferential integration in transcription start sites and regulatory gene regions[133]. These integrations were shown to result in the activation of cellular proto-oncogenes, (LMO2, MDS-EVI1, CYCLIND2, or BMI1) and caused the development of leukemia in 5/19 patients from two X-SCID trials[134,135] and a growth advantage for gene-transduced cells resulting in the occurrence of dominant clones in a trial for X-CGD[136].

Although many AIDS patients develop malignancies during their illness, HIV-1 has not been linked directly to cancer, even though all HIV-infected cells carry randomly integrated proviruses. Lentiviral vectors integrate preferentially in transcribed genes, rather than in transcription start sites and regulatory gene regions and are therefore considered less genotoxic than the gammaretroviral vectors[133]. Furthermore, it was shown that the genotoxic potential of retroviral vectors is strongly modulated by vector design, in particular the vector's enhancer-promoter elements[133] and the LTRs[137]. The new self-inactivating (SIN) LTRs were shown to significantly enhance the safety of both LVs and RVs and alterations of the LTRs had a greater effect on safety than the retroviral insertion pattern[137].

Vaccinia-based vectors

Members of the family of Poxviruses are not associated with malignancy, but may give rise to benign tumors. Vaccinia virus is a ds DNA enveloped lytic DNA virus, but in contrast to other DNA viruses, its life cycle takes place exclusively in the cytoplasm of the infected cells.

Possible effects immune modulation

Immune modulation may increase the amount of infectious particles that integrate and thereby increase the risk for insertional mutagenesis in the treated patient. However, only in cases of germline transmission and insertional mutagenesis after horizontal transmission to thirds would this be considered an increased risk for the environment.

2d. Information about deletions of viral sequences

I Deletions of genes to render viruses replication deficient

II Deletions of genes important for immune evasion

III Deletions of other viral sequences

2e. Information about inserted transgenes or sequences

Background

Transgenes can be categorized according to cellular gene and function. A workable classification therefore would be into genes encoding structural proteins, eg actin or myosin; enzymatic proteins: serum proteases, phosphatases etc; metabolic enzymes: required for amino acid metabolism or nucleotide synthesis; proteins required for cell growth and house keeping; proteins required for cell cycle and cell division; proteins used in DNA replication; membrane proteins: ion channels, G-coupled protein receptors, transporters, etc; proteins enhancing cytotoxic or lytic activity, eg fusogenic membrane proteins[138]; tracking genes such as GFP, luciferases and photoreactive genes; selection genes, eg MGMT; antibiotic resistance genes, such as neomycin (G418); suicide genes, such as TK/ganciclovir[139]; prodrug-activating genes, eg 5-Fluorocytosine/cytosine deaminase[139,140], P-450/ Cyclophosphamide[141,142]; active subunit genes for toxins, eg botulinum toxin, Shiga, and Shiga-like toxins; regulatory genes, transcription factors; growth factors, cytokines, chemokines; immune modulatory molecules, eg CTLA4Ig, B7-1; oncogenes, mutations in tumor suppressor genes. See Table V.

I Toxicity
II Survival advantage, eg cytokine, growth factor, receptor
III Survival disadvantage, eg antigen, replication inhibitor
IV Antibiotic resistance, eg selection gene
V Requires activation, eg tumor suppressor
VI Other, eg deficiency, suicide, marker

Possible effects immune modulation
Of these categories, the genes coding for toxins or cytotoxicity enhancing molecules and the genes that may result in survival advantage, eg oncogenes or mutations in tumor suppressor genes, antibiotic resistance genes and to a lesser extent the regulatory genes are genes that might carry a possible risk for the environment. Transfer of these genes as a result of shedding into the normal population may be increased by immune modulation indirectly (by increasing risk for shedding).

2f. Replication of the viral vector in normal cells
2g. Immune evasiveness

Background
Whether or not the immunomodulation is used depends on the remaining viral evasion mechanisms of the vector after modifications, as well as on the new shielding techniques applied. No matter how well the viral evasion mechanisms function, the viral vector inoculation dose is far larger than any number of viral particles encountered as the result of a natural infection. In addition, here the route of administration and the target organ or tissue is highly important in the determination of whether or not some form of immune modulation should be used. The goal of immune modulation is to allow the vector to reach its target tissue, when its own viral evasion mechanisms are insufficient.

Viral evasion mechanisms present in the vector
I Viral evasion mechanisms
II Structural modifications (shielding or stealth mechanisms) affecting antigenic variability of the vectors

2h. Availability of preclinical models
2i. Information about the tropism, targeting and restricted expression of vectors

Background
The natural tropism of a virus can be restricted if broad to increase safety or enhanced if limited to target more tissues. Vectors have been modified to preferentially target and replicate in specific cells, eg tumor cells or a specific cell type, to minimize the effects on other cells or tissues and increase the efficiency of gene transfer[97]. The role of targeting becomes more important when gene therapy is delivered systemically than upon local (tumor/tissue) injection and issues that need to be addressed when applying systemic approaches include the immune response, single or multiple administrations, and the stability and pharmacokinetics of the complex when injected in the peripheral circulation[97]. For more information, see also: Engineering targeted viral vectors for gene therapy by Waehler *et al*[143].

I Transductional targeting: Surface targeting & Capsid modifications

Background
- Serotype switching[144,145] (eg Ad, AAV), selection of specific variants (AAV)[146-148]
- Pseudotyping: may alter surface receptors and host range/cell tropism (VSV-G/GALV, ecotropic/ amphotropic), immunogenicity of the vector (VSV-G) and immune escape (AAV)[149]
- Engineering of viral envelope, eg designed capsid domains[150]
- Altered way of adsorption/penetration: changed cell attachment (CAR-binding mutants), entry and cell-to-cell spread, different surface antigens/receptors/ fusogenic peptides, proteins or antibodies (indirect targeting) or genetic targeting by introduction of specific sequences, eg RGD[151,152]
- Coating of viral surface with polymers: PEGylation (affects strongly persistence of virus in circulation and biodistribution to distinct organs[153]

II Transcriptional targeting: Genome modifications for targeted replication

Background
- Tissue/cell-specific promoters (note: often too specific, target only a subgroup of cells in a tumor, choose tumor-specific promoters or radiation-activated promoters)[154]
- Targeted replication of oncolytic virus in cancer cells (eg p53)[154]

- Targeted replication in tumors through local activation[7] (eg MMP)[155]
- Targeted transcription by promoter elements that become activated in chemotherapy resistant tumor cells[156], radiation inducible elements[157], hypoxia-response enhancer elements[158], or Cre/loxP[97,159]

III Targeted replication by post-transcriptional regulation of replication of expression

Background
Transgene expression can be detargeted by expression of endogenous tissue-specific miRNAs[160,161]

2j. Information about biodistribution after systemic injection, local injection in a non-immuneprivileged site, local injection in an immune privileged site.

Background
Biodistribution is strongly dependent on the route of administration (see above) and on whether the vector is replication competent or not. Replication of oncolytic vectors can result in a local or distant distribution of infectious virus. From a safety perspective, treatment of large tumor masses with local injection and replication of oncolytic virus would be ideal. However, intratumoral injection therapy is limited to needle-accessible disease sites, whereas intravenous administration allows the potential for viral infection of even non-injectable disease sites and treatment of metastases.

Replication-competent vectors
Without replication, ONYX-015 is cleared from the circulation within 24 h in animal models and in patients after iv administration. DNA titers in plasma declined with a half-life of about 20 min over the first 2 hrs following dosing, after which a plateau was reached for 2-6 hrs, followed by a steady increase in viral DNA, indicating replication[73]. Viral infection of metastatic tumor sites and shedding into the circulation was observed for several weeks in high-dose patients and viral pharmacokinetics were not altered by neutralizing antibody formation[73]. Vaccinia virus is highly stabile in the circulation and intratumoral or systemic injections of Vaccinia virus leads to dissemination to distant tumor sides.

Replication-defective vectors
Biodistribution is here mainly dependent on the route of administration, level of pre-existing host immunity and the total dose of infused vector.

Possible effects immune modulation
Immune modulation may prolong the existence of both replication competent and defective viral vector in systemic circulation or in local tissues. This may result in a broader biodistribution pattern and increased off-target transduction, in particularly the liver, and increase the risks of co-infection and recombination.

2k. Information about persistence after *in vivo* administration

2l. Information about mutation and recombination after *in vivo* administration

Background
Mutation and recombination are events that occur more often in RNA viruses than in DNA viruses due to the absence of proofreading mechanisms. Other factors of influence on the rate of mutations/recombinations involve the viral strain, the transgene position and level of expression and the host cell. Selection agents, eg ganciclovir and acyclovir, may also put strain on the viruses to increase mutation rate. A high vector inoculation dose will increase the likelihood of co-infection of a cell with a related wt virus, since more cells are infected.

I Is the vector replication competent or replication deficient?

II What is the incidence of mutation and recombination of the parental virus?

III What is the incidence of mutation and recombination of the vector *in vitro/in vivo*

IV Information on complementation or missing functions in the vector by the wt virus

V Other treatments that may influence recombination/mutation (eg ganciclovir)

Possible effects immune modulation
Immune modulation may increase the number of particles available to infect target cells, increase the vector persistence and thus the time for interaction with wt virus and may affect the viral life cycle and biodistribution. Immune modulation can also

potentially increase the risk for a new primary infection with wt virus or may result in reactivation and active replication of wt latent viruses, increasing the risk for co-infection. Replication-defective vectors are used in most cases to deliver a transgene to a target tissue, but since the vector cannot replicate, the vector particle dose has to be relatively high to obtain good transduction efficiency. Immune modulation may be required to increase initial transduction efficiency and induce tolerance against the transgene. The time needed to bridge with immune modulation may be protracted, but with relatively little effects on recombination. Replication-competent vectors are mostly used in treatment of tumors and a relatively low vector dose can be sufficient to allow local and tumor cell-sensitive replication. Immune modulation may be given for a short period of time only, since vectors are actively replicating, may still have a considerable effect on recombination.

2m. Information about possible toxicity (as a result of either the vector itself or the encoding transgenes)
2n. Environmental shedding

Background
Presence of viral sequences in tissues or specific organs may increase the risk for environmental shedding. Body fluids can be a source of (replicating) viral vector and inadvertent exposure to blood, urine, seminal fluids (AAV)[49] or needlestick accidents may result in transmission of vector. In the wild, rodents are associated with outbreaks of exanthematic lesions caused by Vaccinia virus in humans and dairy cattle and have been shown to serve as a virus reservoir[162,163]. It was shown that for 20 days after wt Vaccinia exposure, infectious particles could be detected in feces, whereas viral DNA could be detected for up to 60 days[163]. Exposure of healthy mice to excreta of Vaccinia infected mice resulted in horizontal transmission[164]. Thus, shedding and long-lasting stability of Vaccinia virus in murine feces requires careful handling of Vaccinia infected laboratory animals and their excreta should be considered a potential source of transmission. In addition, it is highly recommended that all laboratory personnel working with VACV be vaccinated[79]. Direct contact with VACV vector injection sites was shown to result in transmission to secondary and even tertiary recipients[52,165].

I Respiratory
II Body fluids
III Feces
IV Direct contact

Possible effects immune modulation
Immune modulation can prolong the half-life of viral vectors in circulation and change biodistribution. As a result, shedding may be observed for a longer period of time and from multiple different excreta.

2o. Horizontal transmission
2p. Safety back-up

Background
Does the vector system contain a suicide gene (natural or engineered) or can adverse events be terminated by the use of anti-viral agents.

I Is a suicide gene present?
II Are the vectors sensitive to anti-viral agents? (See also Table IV)

Step 3: Information about production of the vector

Required information
An overview of the production process, ie the origin of the producer cell lines, the genetic components of these cell lines, required to complement virus production, quality control measures and criteria used to reject a batch) are required.

3a. Which producer cell lines are used?
3b. Which viral functions are provided by the cell lines?
3c. Which quality control measures are used?
3d. Which criteria are used to reject a batch?

PART C: IMMUNITY-RELATED ISSUES

It must be kept in mind that although discussed separately, many of these factors are interconnected and influence each other, such as age and the development of immunity.

Step 1: What is the level of host immunity?

Required information
All relevant medical information about the patient, affecting the host immune system, such as genetic factors and patient medical history pointing or suggesting a compromised immune system.

1a. Does the patient have a functional immune system: Is the patient immune-competent or (relatively) immune-deficient. If the patient is immune impaired, what is the extent of the immune deficiency?

Background
A malfunctioning or absent immune system cannot defend the host against the invading organism and even a harmless or attenuated virus can result a pathogenic response, a disseminated infection, with possible lethal outcome and spreading to the environment. If the functional immune sstem is (relatively) impaired, this could potentially influence the viral life cycle, the virulence of the vector, the biodistribution, persistence and shedding. This may affect in particular the choice for a replication competent or replication deficient virus. Whether immune modulation will be used in patients with an already defective immune system depends on the depth and duration of the immune deficiency. It was shown that for an effective secondary anti-VACV response, the antibody response is obligatory, whereas the CTL response is non-essential[166]. However, most other viral antigens are T-dependent, which means that for an optimal B-cells response interaction with T cells is required[15].

I impaired innate immune system
II impaired adaptive/cellular immune system
  I primary immune deficiency (PID)
  II secondary immune deficiency (AIDS)
  III induced transient immune suppression (chemo/radiotherapy)

Possible effects immunomodulation
The risk for adverse events (infections, spreading, recombination, increased virulence) can be increased in patients with a defective immune system, irrespective of the cause, although the sensitivity for specific types of infections may differ. Immune modulation can affect the outcome of patients with an otherwise functional immune system and result in prolonged presence of vector.

1b. Does the patient have a pre-existing condition (co-morbidity) affecting immunity, such as a mental or physical disability or for example diabetes?
I Due to the presence of any or more of the factors in Table VIII
II Due to increased risk of exposure to a specific virus (eg hospital personnel, laboratory personnel)[167]

1c. Does the patient have proven pre-existing or cross-reacting immunity (presence of antibodies) against the viral vector or is it expected that the patient may have pre-existing or cross-reacting immunity against the viral vector?

Background
The functional immune system will interact with any viral vector, but the velocity and the magnitude of the response dictate the clinical efficacy of the treatment: A primary immune response against the vector will be relatively slow. If the transgene is recognized as a neoantigen, and has been administered to the patient before, eg factor VIII or enzyme replacement therapy, an adaptive immune response against the protein will result in a CTL-mediated response and formation of neutralizing antibodies through a CD4+ T-cell facilitated mechanism[168]. The development of neutralizing antibodies, which bind to the surface of viral particles, prevents viral binding to cellular receptors required for cell infection.

The non-neutralizing antibodies bind to viral particles, fixing the complement pathway and are less important here. The secondary response due to preexisting immunity against the viral vector or the transgene can result in a rapid onset and clearance of the vector and foreign protein, resulting in low clinical efficacy and may cause considerable side effects, expecially when the response against the vector becomes unbalanced. Although neutralizing antibodies or memory

T-cell responses can also make readministration impossible, the neutralizing activity may also prevent spread of replicating virus, adding to safety: in immunocompetent animals, preexisting immunity to the vector did not affect vector antitumor efficacy following intratumor injection of the vector, but it markedly reduced spillover of the vector to the liver and lungs[169] and decreased toxicity of the treatment[170]. Oncolytic virus may locally replicate and infect tumor cells, but spread and replication in less permissive tissues may be relatively contained. The presence of absence of pre-existing immunity is more important when multiple viral vector doses are required (eg cancer treatment).

I previous infection with the wt virus or a closely related virus
II previous vaccination with the wt virus or closely related virus
III previous treatment with this viral vector or a similar vector
IV previous contact with the transgene, eg enzyme replacement therapy
V confirmed presence of neutralizing or cross-reacting antibodies (make sure the test is not inadequate due to low assay sensitivity)

Possible effects immune modulation

If the answer to any of the above is Yes, it is likely that the half-life of the vector upon injection will be decreased and therefore the efficacy of the treatment. Re-evaluation will be necessary and the treatment protocol may have to be adapted accordingly, eg increased vector dose, different serotype of vector or immune modulation may be indicated. Each of these choices will affect the environmental risk and re-evaluation has to start from the beginning. Immune suppression targeting specifically pre-existing immunity includes cyclophosphamide (CY), targeting B-cells and CD4+ T-cells and Cyclosporin A (CsA). Although transgene expression is usually somewhat prolonged, there appears to be no effect of CsA on neutralizing antibody levels. CY increases anti-tumor efficacy (independent of the presence of NAb), likely by decreasing not only the anti-vector response (and thus may increase the vector response) but may also by boosting the host anti-tumor immunity[169]. Preexisting immunity can prevent spread from oncolytic virus and although CY treatment decreases NAb, it does not affect the NAb-effect on containing spread and replication of oncolytic virus to other organs, suggesting some level of NAb can be protective.

1d. Is the patient a known or suspected carrier of a virus, similar or closely related to the viral strain, used for the vector (presence of viral nucleic acids)?

I Does the patient have a history of infection(s) with wt virus used as viral vector?
II Does the patient have a current infection or inflammation?

Background

Acquired coexistent pathologies, eg infections, can act as inflammatory adjuvants at the time of gene transfer[57]. This can have two opposite effects: the overstimulated innate responses activated by a concurrent infection may result in a more pronounced host immune response against the vector, or can result in more severe (lethal) side effects. In addition, injection of viral HSV vectors may reactivate or recombine with endogenous latent HSV, and similarly Ad vectors with endogenous latent wt Ad. Even more, previous encounters with the same wt virus that is used as a vector may result in an accelerated and enhanced immune response against the vector, decreasing the efficiency (see pre-existing immunity. If the subject is a carrier of latent wt virus, closely related to the intended vector, treatment with the vector may result in reactivation from latency (eg HSV, AAV, Ad), recombination, mutation or reversal to wild-type status. Current infections or inflammatory processes should be treated and resolved before proceeding, after which the risks can be re-evaluated. An exception to this may be the treatment of HIV carriers with lentiviral vectors, where ongoing HIV infection is a requirement for the treatment *per se*, however, other opportunistic infections should be treated and cleared before gene therapy.

Possible effects immune modulation

Immune modulation may increase the risk for reactivation of latent wt viruses and recombination with vector-type virus, it may affect spreading, biodistribution and shedding. Immune modulation may increase the severity of ongoing infections or inflammatory processes and give rise to serious side effects in the patient. However, the latter is not relevant for the environmental risk assessment.

Step 2: What type of immune modulation is used?

Required information
All relevant information concerning dose, duration of treatment, and its relation to the depth of immune suppression, in particular T-cell immunity.

Background
As discussed extensively in appendix B, there are many types of immune modulation, each targeting different systems with more or less specificity. The rationale behind the use of immune modulation in gene therapy is clear (increased vector efficiency), but it should outweigh the possible risks, such as an increased risk for other infections, the likelihood of recombination, effects on the viral life cycle, and the prolonged persistence of vector in the body and spreading through the circulation (biodistribution) and shedding. In addition, often immune suppressive treatments are administered for primary tumor-treatment and not with the intention to prolong vector existence, but this may nevertheless have the same effect.

2a. What is the type of immune modulation(s) used?
Stratify according to 4 risk categories:

I Systemic immune suppression with drugs resulting in total myeloablation, severe myelosuppression or T-cell depletion (in particular ATG or Alemtuzumab)
II Specific targeted immune modulation with agents blocking co-stimulation
III Steroids
IV Other

Possible effects of immune modulation
Systemic immune suppression affects multiple routes of immunity, and may in some cases even lead to bone marrow suppression, whereas specific immune suppression, even if delivered systemically, affects a single factor. Even more, specific immune suppression, for example with CTLA4Ig, can decrease the immune response towards the vector, without increasing the risk for infection. Long-lasting immune suppression can significantly increase the risk for infection, whereas transient immune suppression may allow the vector to infect the cells, but not increase the risk for infection. The effect of the different immune modulatory agents and their effect on infection risk are summarized in Table III.

2b. What is the dose (single or multiple) and duration of the treatment(s)?

2c. What is the anticipated (intended) level and duration of immune suppression achieved with this type of immune modulation? Ie, is the immune suppression intended to be transient or long-term.

2d. What is the rationale behind the choice of immune modulation?

I Based on induced immune response against the vector
II Based on animal studies
III Other

Step 3: What is the effect of this particular immune modulation regimen on the patient's risk of infection?

Required information
All relevant data indicating increased risk of infection in relation with the depth and duration of the type of immune modulation used (For further information, check Table III)?

Background
Increased infection risk, may increase the chances of two related viruses (ie a wt virus and the vector) to interact with each other. The risk of infection is determined by a number of variables, some of which are treatable (eg predisposing diseases or particular treatments), whereas others cannot be influenced (eg age). Based on experience with immune compromised patients and human vaccinations, the infection risk in general appears to be considerably higher in specific groups of people. These groups have been summarized in Table VIII. In addition, some occupational exposure can result in an increased risk for specific infections.

3a. Is the immune modulation used associated with an increased risk for primary infection?
3b. Is the immune modulation used associated with reactivation of latent viral infection?

Possible effects immune modulation
Immune modulation can affect the risks for multiple (simultaneous) infections, especially in groups of patients who already display an increased risk of infection.

Step 4: Is the patient subject to other treatments that may affect the immune modulation facilitated gene therapy treatment?

Required information
This involves all relevant information about additional treatments which may interfere with the functioning, the biokinetics and halflife of the immune modulatory agents, directly affect the immune system itself, either as an intended effect or as a known side-effect, or other systems indirectly affecting the immune system, as well as treatments that may influence the biodistribution, persistence, viral life cycle, recombination and shedding of the viral vector.

4a. Which treatments are (possibly) used, at what dose and duration?
4b. Do these treatments in any way affect the functioning, biokinetics and half-life of the immune modulatory agents?
4c. Do these treatments affect in any way the patient's immune system?
4d. Do these treatments influence the viral vector, by interfering with biodistribution, persistence, viral life cycle, recombination and shedding?

Step 5: Does the immune modulation in any way affect viral vector biodistribution and persistence?

Required information
Provide any scientific information (and references to) concerning relevant animal or clinical studies where this type of immune modulation is used in combination with gene therapeutic applications. What are the relevant (pre)clinical studies and why are the relevant here: which organs are involved and how long can vector be measured and by which techniques, eg. Semi-qPCR, RT-PCR, nested PCR, Taqman, PCR/Southern; tracking genes, eg EGFP, luciferase, LacZ; Histology; FISH; ELISA; transgenic protein/gene expression; cell culture, etc.

5a. What are the relevant animal/clinical studies?
5b. How is biodistribution of the vector affected by immune modulation?
5c. How was biodistribution and persistence of the vector measured?
5d. How long were biodistribution and persistence measured and up till what time were samples found to contain evidence of viral presence?

Step 6: Does immune modulation affect the likelihood of recombination or reassortment of the viral vector?

Required information
Provide scientific information (and references), if available, on recombination events in *in vitro* and *in vivo* animal studies.

6a. What are the relevant studies?
6b. How are recombination or reassortment affected?

Step 7: Does immune modulation affect the risk for shedding and transmission of the viral vector?

Required information
Provide relevant scientific data (and references) on shedding from animal and clinical studies. Which sites were sampled (eg injection site, respiratory tract, excreta (urine, stool, semen), bandages, patient's (hospital and home) environment, which methods were used to obtain the samples (blood, serum, swab, etc) and to confirm shedding (PCR, cell culture, etc) and how sensitive are these tests. At which time point was shedding measured and up till what time and how often.

7a. What are the relevant pre(clinical) studies?
I How was shedding measured?
II From which sides was shedding measured
III How often and how long was shedding measured?
IV How was secondary/tertiary transmission measured?
V Were any negative effects of secondary/tertiary transmission documented?

This ERA consists of three parts, as discussed above: Part A: General and treatment-related issues; part B: Vector-related issues; and part C: Immunity-related issues. A fourth part D is not provided here, but would ideally contain all aspects as discussed above and allow an integral evaluation of the risks, according to directive 2001/18 (effects on human health, medical practice, animal health, veterinary practice, population dynamics) and the assessment of the overall environmental risk. However, all relevant information needed for such an individual risk evaluation is provided in different parts of this report and background reading can be found in the appendices.

3.3 ERA SAMPLE CLINICAL TRIAL

Example of a fictional ERA with the AAV1-LPL^{S447X} vector.

PART A: GENERAL AND TREATMENT-RELATED ISSUES

Step 1: Describe the patient population

1a. How many patients are included in the clinical trial?
Estimated enrollment 8.

1b. What is the age range of the patient population?
Over 18 years

1c. Describe the disease (genetic, cancer, etc).

Lipoprotein lipase (LPL) is the principle enzyme involved in the clearance of triglycerides from plasma. Severe LPL deficiency is a disorder affecting approximately 5000-10000 individuals in the Western world. The prevalence of familial LPL deficiency is approximately one in 1,000,000 in the general population. These patients present with colicky pain, eruptive xanthomas, growth retardation and recurrent acute pancreatitis, resulting intensive care admissions, diabetes or death. Approximately 25% of affected children develop symptoms before age one year and the majority develops symptoms before age ten years. There is currently no specific therapy available other than severe reduction of dietary fat, to 20 grams/day or less, which is impossible to comply with in the long term. Lipoprotein lipase (LPL) is the key enzyme in the metabolism of triglyceride-rich lipoproteins and is mainly produced in fat tissue, skeletal and heart muscle. Enzymatic activity of LPL mediates hydrolysis of triglycerides in chylomicrons and very low density lipoproteins (VLDL), resulting in the production of free fatty acids for either energy-expenditure or –storage. Consequently, these triglyceride-rich lipoproteins are rapidly cleared after every meal and the triglyceride levels in the circulation are reduced. LPL deficiency is an autosomal recessive inherited condition caused by homozygosity or compound heterozygosity for mutations in the LPL gene.

1d. Does the disease itself affect the immune responses of the patient?
No

1e. What are in/exclusion criteria

Inclusion criteria:

- Being diagnosed with LPLD defined as:
- Confirmed homozygosity or compound heterozygosity for the mutations in the LPL gene, resulting in LPL deficiency
  - Having a post heparin plasma LPL activity of < 20% of normal or a well defined mutation for which it is documented that the LPL mass and activity are within the limits described above
  - Having a history of pancreatitis
  - Having fluctuating TG concentrations with median fasting plasma TG concentrations > 10 mmol/L
- Being in good general physical health within the opinion of the investigator:
  - No other clinically significant and relevant abnormalities in the medical history, which could interfere with the participation to the study
  - No clinically significant abnormalities at the physical examination, which could interfere with participation to the study
  - No clinically significant abnormalities at the routine laboratory evaluation performed prior to the trial
- Women of non-child bearing potential or with a negative pregnancy test
- Non breast feeding women
- Women using appropriate contraceptive (if relevant) and their partner using barrier contraception 2 weeks before starting immunosuppressive therapy
- Men practicing barrier birth control and their partner using appropriate contraception.
- Willing to fully comply with all study procedures and requirements of the trial such as restrictions to a low-fat diet.

Exclusion criteria:

- Having a chronic inflammatory muscle disease
- Any current or relevant previous history of serious, severe of unstable physical of psychiatric illness, any medical disorder that may make the subject unlikely to fully complete the study, or any condition that presents undue risk from the study medication or procedures based on the investigator's opinion (eg. malignant neoplasia)
- Active infectious disease of any nature, including clinically active viral infections
- Having one of the following outcomes from the blood screening tests after appropriate correction due to the presence of chylomicronemia:
  - Platelet count < 100 x 10^9/L
  - Hemoglobin < 6.2 mmol/L

- Liver function disturbances (bilirubin > 1.5 x normal, ALT > 2x upper limit of normal)
- CPK > 2x ULN
- Cockcroft-Gault estimated creatinine clearance < 50 cc/min
- PT and PPT outside normal range or not determinable unless judged as acceptable for the subjects by the investigator
- Having a positive test for HIV, Hepatitis B, Hepatitis C or being positive for tuberculosis

- Obesity defined as a body mass index (BMI) > 30 kg/m^2
- Having a recent history of alcohol or drug abuse, eg barbiturates, cannabinoids and amphetamins, and the subject is positive in a urine screen for drugs of abuse
- Using anti-coagulants
- Participation in another clinical trial or receipt of any other investigational drug within 30 days of screening or planning to participate in another clinical trial during the course of the study, except observational studies
- Subjects which cannot be treated with immunosuppressive medication or steroids
- Known to be allergic to any constituent of the therapy (including immune suppressors) or having a condition that prohibits the use of therapy
- Received previous treatment with AAV1-LPL or other gene therapy investigational product
- Requiring a post-heparin plasma LPL activity test for diagnostic confirmation and having a history of heparin-induced thrombocytopenia or other heparin-related complications

1f. What are the primary and/or secondary endpoints of the study?
The primary endpoint is a reduction in median fasting plasma TG concentrations at week 12 of levels < 10 mmol/L or a reduction in TG levels of > 40%. Secondary endpoints include reductions in chylomicrons and/or the chylomicron/TG ratio at 12 weeks, the biological expression of the LPL^{S447X} transgene product, the safety profile, a reduction in the incidence of pancreatitis and shedding of viral the vector at week 14.

Step 2: How is the viral vector administered?

2a. What is the route of administration (ROA)?
(systemic: sc, ip, iv, ia; or local: intratumoral, intralesional, intrathecal, intrapulmonary.)
Intramuscular delivery in the upperlegs

2b. What is the way of administration? (injection, inhaler/spray, other)
A single series of multiple injections (40-60) of 500 µL each, was previously shown to be well tolerated in a clinical trial (Stroes *et al.* 2008).

2c. What is the dose (pfu, vp, ip) and timing of admini tration (single dose, multiple doses, time in between treatments)?
1x10^{12} genome copies per kg body weight. A previous study (Stroes *et al.* 2008) showed that higher doses showed better results in terms of clearance of fasting plasma trigliceride levels.

2d. Is the target organ an immune-privileged site or non-immune privileged site?
Non-immune privileged site

Step 3: Information about other treatments applied to the patient population

3a. Treatments directly affecting the immune system
None

3b. Treatments affecting pharmacokinetics of viral vectors
None

3c. Treatments affecting pharmacokinetics of immune modulatory agents
None

3d. Other
None

Step 4: Information about the patient's environment

4a. Hospitalization-related information
Patients are nursed in a controlled environment where a dedicated facility appropriate for gene therapy procedures is available, with limited monitored access for experienced and fully trained personnel, according to legal regulations.

4b. Housing-related information, including household information, nursing-home
This section is patient specific and should be evaluated on a case by case basis. Here no particular circumstances apply.

4c. Out-house activities, such as profession, military, kindergarten/schools, sportsclubs, etc
This section is patient specific and should be evaluated on a case by case basis. Here no particular circumstances apply.

4d. Animal contacts (occupational or recreational)
This section is patient specific and should be evaluated on a case by case basis. Here no particular circumstances apply.

Step 5: Which protective measures are utilized to prevent transmission?

5a. To healthcare personnel
Infection precautions recommended for adenoviruses should be maintained, because transmission of AAV may be similar to that of adenovirus and is most probably via the respiratory or gastrointestinal tract. In addition, prevention of adenovirus, herpes virus, or vaccinia virus infections may be useful, because propagation of AAV is dependent on coinfection with these helper viruses. Infection control of adenovirus is difficult. Adenovirus can persist for up to 30 days on surfaces and are not eliminated by standard detergents, alcohol or chlorhexidine gluconate. Handwashing may therefore not suffice. However, the use of sodium hypochlorite (10% bleach) is effective in killing the virus (Evans and Lesnaw, 2002). Contact with bandages and the site of injection should be minimimized to prevent transmission. Droplet and contact precautions, including personal protective equipment and hypochlorite in case of spilling, are required during and shortly after administration and during hospital admission standard precautions and the use of personal protective equipment (surgical gloves, mask) will be maintained. Equipment from the patient's room will be discarded, if appropriate and waste will be disposed of as regulated medical waste.

5b. To household members

5c. How long will these measures be continued, eg before, during and after therapy
Airborne and Contact Precautions will remain in effect until two consecutive assays for the appropriate body site or fluids are negative for the vector.

5d. How is compliance with the measures assessed
Hospital personnel, patients and family members will all receive appropriate instructions.

PART B: VECTOR-RELATED ISSUES

Step 1: Information about the wild type virus

1a. Which wild type virus is used as a backbone for the vector.
The adeno-associated viruses (AAV) are small, non-pathogenic, single-stranded DNA viruses and naturally replication-defective. The most commonly used rAAV vector for gene therapy is based on AAV-2. However, due to the high level of immunity against AAV-2 also the use of other AAV serotypes is explored, including AAV-1. The choice of AAV serotype is strongly determined by their tropism. The natural tropism of AAV-1 for muscle tissue, CNS and liver tissue and the decreased immunogenicity of this serotype, make this serotype ideal to serve as a viral vector to target muscle. Here, a recombinant AAV, produced in a baculovirus sytem is used, pseudotyped with AAV-1 capsids and containing AAV-2 inverted terminal repeats.

1b. Infectivity of non-replicating cells
AAV can infect both replicating and non-replicating cells.

1c. Integration into host genome
In the absence of helper virus, AAV-2 can become latent and integrate site-specifically into chromosome 19q13.4. This specificity of integration is determined by the presence of the ITRs and the rep gene.

1d. Virulence and pathogenicity
AAV is a non-pathogenic virus and although infection with wild type AAV may occur, this is not associated with human disease. Approximately 85% of adults have antibody against AAV and seroconversion usually occurs in childhood. The route of infection is presumed to be, like adenoviruses, respiratory or gastro-intestinal.

1e. Host-range: Human/Animal, Broad/Restricted
AAVs can be found in humans, non-human primates and a broad range of other animals.

1f. Tissue tropism
The tissue tropism of AAV is broad and includes muscle, CNS and liver, but depends on the serotype and expression of receptors and co-receptors. For example, AAV-2 gains entry into target cells through binding to heparan sulfate proteoglycan and one or more co-receptors including anb1 and anb5 integrins, FGF-R1, HGF-R and the lam-

inin receptor and targets kidney, liver, muscle, lung and CNS tissue. AAV-1 tropism is restricted to muscle, CNS and liver tissue and uses sialic adic as its primary receptor.

1g. Biodistribution

Biodistribution data of AAV1 in mouse models showed short-term vector leakage from intramuscular injection sites into the circulation, followed by liver-mediated clearance. AAV1 pseudotyped AAV2 vaccine vectors showed that after intramuscular injection in rabbits biodistribution and persistence depended on the vector dose and were most common at the injection site and highly perfused tissues, eg liver, iliac lymph nodes, spleen and testes, but not in sperm. One liver sample tested positive for integration, all other samples persisted as unintegrated episomal concatemers (Schnepp *et al.* 2006).

1h. Persistence

Most AAV serotypes persist episomally and can remain present in non-dividing cells for extended periods of time. AAV2 can integrate in the genome and is usually found as a provirus integrated into chromosome 19 of the host cell genome, where it remains latent until helper viruses supply missing proteins and genes, required for successful replication.

1i. Cell lysis and lateral spreading

Naturally occurring AAV serotypes, in particularly AAV-2, can become widely disseminated following primary infection in children (Chen *et al.* 2005). The most probable route of infection is through the respiratory system, after which primary infection occurs in association with a helper virus, most commonly adenovirus. Replication with the help of adenovirus, results in the formation of new AAV particles, which may spread through the oropharynx or bloodstream to distant sites. Even widespread dissemination is not associated with any clinical disease or pathology (Chen *et al.* 2005).

1j. Innate immune response

AAV is able to evade innate host immune surveillance, due to lack of pathogen associated molecular patterns (PAMPs), preventing the activation of Toll-like receptors (TLRs). The host response against wild-type AAVs is therefore at least partially determined by coinfection with a helper virus, which induces the innate immune response and facilitates the immune response against AAV. Other parameters that determine the host immune response against AAV are pre-existing immunity, route of administration, inoculation dose, serotype and its ability to infect APCs.

1k. Viral clearance

AAV clearance is predominantly cytotoxic lymphocyte mediated.

1l. Horizontal transmission

Transmission of rAAV is most likely to be similar to that of adenoviral vectors and may occur through droplets from the respiratory tract or body fluids, such as stool and urine.

1m. Vertical transmission

Vertical transmission of AAV has not been observed in any preclinical or clinical study. Although vector genome from different AAV serotypes, eg AAV-1, AAV-2 and AAV-8 could be transiently detected at low levels in the gonads or semen after administration to several animal species, including mice (Rip *et al.* 2005; Jakob *et al.* 2005), rabbits (Favoro *et al.* 2009) and non-human primates (Toromanoff *et al.* 2008), this did not result in germ-line transmission (Van Amersfoort *et al.* 2007; 2008) and the overall risk was considered very low. Importantly, the appearance of vector genome copies in semen was shown to occur in a dose-dependent and time-dependent fashion (Schuettrumpf *et al.* 2006). Similarly, in two phase I/II clinical studies for lipoprotein lipase deficiency with an AAV-1 pseudotyped vector (Nierman *et al.* 2007) and with an AAV-2 vector for hemophilia (Manno *et al.* 2006) vector sequences were detected in semen. In the latter, however, vector appearance and clearance from semen was found to be not dose or time-dependent, but rather depended on the age of the patients, with younger men displaying earlier clearance than older men. In rabbits, clearance of vector sequences from the motile sperm fraction was more rapid than from total semen, and there was no evidence of transduction of early spermatogonia (Schuettrumpf *et al.* 2006). Semen fractionation in humans demonstrated the absence of vector sequences in motile sperm (Manno *et al.* 2006).

1n. Genetic stability/Recombination

No data are available on the genetic stability or recombination of naturally occurring AAV.

1o. Availability of anti-viral treatment

Currently not available.

1p. Physical and chemical stability

AAV has is stable against heat and pH changes and has

a relative solvent resistance. It is not resistant to various solutions containing 10% bleach.

1q. Immune evasiveness

AAV lacks PAMPs, as a result of which they cannot activate the TLRs . Although AAV-2 can infect DCs through binding of Heparan Sulfate Proteoglycan (HSPG), a post-entry block inhibits successful transgene product expression and upregulation of co-stimulatory molecules and MHC classes I and II. As a result, AAVs are not able to induce maturation of human DCs and production of type I IFNs.

Step 2: Information about the viral vector

2a. Is the vector replication-competent?

Previous vector production was performed using plasmid transfection of the HEK293 cell line. Upscaling of the vector production required modification of the production system. For the current AAV1-LPL^{S447X} vector, insect cells are infected in suspension culture with three separate recombinant baculoviruses containing the essential genes (P5 for Bac.VD88, Bac.VD84 and Bac.VD43 encoding *rep*, *cap* and LPL^{S447X}) for AAV vector production, thus eliminating the transfection process (Twisk *et al.* 2007; Gaudet *et al.* 2008). The vectors are purified using affinity chromatography. All essential viral genes are provided by the baculoviruses, as are the helper functions of Ad. The resulting AAV-LPL vector is replication deficient. For more information, see also part B, step 3.

2b. Can the vector infect non-replicating cells?

Yes.

2c. Can the vector integrate into the host genome?

The vector remains usually present as an episomal concatemer, but it cannot be excluded that the vector may integrate at a very low level in the host genome.

2d. Information about deletions of viral sequences

I Deletions of genes to render viruses replication deficient

II Deletions of genes important for immune evasion

III Deletions of other viral sequences

AAV requires the help of helper viruses, such as Herpes Virus, Adenovirus or Vaccinia for succesful replication. In absence of these viruses, AAV is replication deficient. AAV contains 2 genes, *rep* and *cap*, which encode polypeptides necessary for replication and encapsidation. Removal of all internal viral coding sequences of the wild-type virus and replacement by a therapeutic gene renders the recombinant AAV completely replication deficient. Here, expression of the LPL variant LPL^{S447X} is under control of the inverted terminal repeat sequences of AAV-2, whereas the capsid proteins are provided by AAV-1. The choice for AAV-1 capsid proteins is twofold, 1) it is less immunogenic than AAV-2; and 2) it has increased specificity and efficacy of transduction of muscle cells.

2e. Information about inserted transgenes or sequences

I Toxicity

II Survival advantage, eg cytokine, growth factor, receptor

III Survival disadvantage, eg antigen, replication inhibitor

IV Antibiotic resistance, eg selection gene

V Requires activation, eg tumor suppressor

VI Other, eg deficiency, suicide, marker

Lipoprotein lipase (LPL) plays a central role in human lipid homeostasis and energy metabolism. The main function of this enzyme is the 1) hydrolysis of plasma triglycerides (TGs) and 2) clearance of atherogenic remnant lipoproteins from the circulation (Rip *et al.* 2006). The protein is mainly expressed in skeletal and heart muscle and adipose tissue. Loss of the LPL gene or loss-of-function mutations result in hyperlipoproteinemia. The S447X polymorphism is found in ~20% of the general population and results in the production of a truncated protein. The LPL^{S447X} transgene is based on the finding that this LPL variant, with a gain-of-function mutation, is associated with beneficial effects on lipid homeostasis and atheroprotection (Wittrup *et al.* 1999) and associated with protection against cardiovascular disease. Overexpression of the LPL variant is not associated with any known toxicity, but expression of the gene in the normal population appears to be associated with a decreased risk for cardiovascular disease. The transgene is transferred to patients with severe LPL deficiency (category VI) and does not result in any known survival advantage or disadvantage of transduced cells. It does not confer antibiotic resistance or require activation. Transfer of the LPL^{S447X} variant to LPL-/- mice resulted in more effective rescue in terms of longevity and lipoprotein metabolism, compared with the transfer of wild-type human LPL (Ross *et al.* 2005).

2f. Replication of the viral vector in normal cells

The AAV1-LPL^{S447X} vector is locally injected through multiple injections in the skeletal muscle, where the LPL

protein becomes active. The LPL protein is expressed locally and transported to the capillary endothelium where it binds to chylomicrons and VLDL. Since the vector itself is replication deficient, it will not replicate in any cell without the help of a wild-type AAV and the presence of a helper virus to complement missing proteins and genes.

2g. Immune evasiveness

I Viral evasion mechanisms present in the vector

II (Structural modifications, shielding or stealth mechanisms) affecting antigenic variability of the vectors

AAV-1 is naturally innate immune evasive as it does not transduce and activate antigen presenting cells, due to lack of pathogen-associated molecular patterns (PAMPs. However, adaptive immune responses may occur towards immunogenic epitopes of the capsid or the transgene product. Whereas the capsid is solely delivered as a protein from an exogenous, non-self renewing source, the transgene product is endogenously produced in the host in a renewable manner (Vandenberghe and Wilson, 2007).

2h. Availability of preclinical models

LPL-/- cat (Ross *et al.* 2006) and mouse models (Rip *et al.* 2005) are available. It was shown in the LPL-/- mouse model that a single intramuscular administration of adeno-associated virus (AAV) serotype 1 vector, encoding the human LPL^{S447X} variant, resulted in complete, long-term normalization of dyslipidemia in LPL-/- mice (Rip *et al.* 2005) and that in cats injection at two sites, greatly limiting the amount of transduced muscle, was sufficient to completely correct the dyslipidemia (Ross *et al.* 2006).

2i. Information about the tropism, targeting and restricted expression of vectors

I Transductional targeting: Surface targeting & Capsid modifications

II Transcriptional targeting: Genome modifications for targeted replication

III Targeted replication by post-transcriptional regulation of replication of expression

The AAV-LPL^{S447X} vector is pseudotyped with AAV-1 capsid proteins and contains the ITR of AAV-2. It was shown in a mouse study that transduction of muscle is superior by AAV-1 compared to AAV-2 and that immune responses against AAV-1 after intramuscular injections are less pronounced than with AAV-2 (Arruda *et al.* 2004). Local diffusion of vector in muscle was found to be limited and although vector sequences could be transiently detected in all body fluids measured, expression of the transgene was found to be restricted to muscle tissue (Rip *et al.* 2005). Whole blood samples tested positive for viral DNA until day 28, but viral DNA in plasma was rapidly cleared (<3–4 days). On day 7, vector DNA was detected mainly in the injected muscles, spleen, liver, inguinal lymph nodes, marrow and gonads. Levels of vector DNA in the various organs and tissues declined with time and were dependent on the vector dose. On day 28 and day 90, vector DNA was detected only in the injection sites, liver and inguinal lymph nodes and viral DNA levels in gonads were just above background levels (Rip *et al.* 2005).

2j. Information about biodistribution after systemic injection, local injection in a non-immune privileged site, local injection in an immune-privileged site.

In LPL-/- mice, shortly after intramuscular administration of AAV1-LPL^{S447X}, vector DNA was detected in muscle, but there was also considerable leakage into the circulation, liver and gonads. AAV DNA sequence also accumulated in lymph nodes close to the injection site, indicating drainage via the lymphatic system (Rip *et al.* 2005). Expression of the LPL protein was only detected in the injected muscles, but not in liver, heart or adipose tissue (Ross *et al.* 2004). In a non-human primate model, it was shown that up to 34 months after im injection, rAAV-1 vector copy numbers were high at the site of injection with little diffusion throughout the muscle, resulting in clusters of locally transduced cells (Toromanoff *et al.* 2008). It was shown by using a replication assay with a sensitivity of $5x10^2$ infectious particles/mL of serum, that the number of infectious AAV-1 particles in serum was highest at 6 hours after im delivery and completely negative within 7 days of injection; using a PCR with a sensitivity of 350 vg/mL of serum, rAAV-1 particles could be detected for up to 1 month in serum; using Southern blotting of PCR amplicons, the detection limit was lowered to ~$3x10^{-5}$ copy/diploid genome and positive samples were found in liver, draining and distant lymphnodes, the gonads, spleen, kidney and PB-mononuclear cells up to 34 months after injection (Toromanoff *et al.* 2008). No immune response was observed against rAAV-1.

In a phase I/II clinical trial with AAV1-LPL^{S447X} it was found that after local intramuscular delivery of $1x10^{11}$ or $3x10^{11}$ gc/kg vector sequences could be detected at high levels in muscle biopsies, but also at low levels in all body fluids tested, including serum, saliva and urine (Nierman *et al.* 2007).

2k. Information about persistence after *in vivo* administration

In LPL-/- mice, persistence of AAV1 vector DNA was limited to the injected muscle and draining lymph nodes, and spread to reproductive organs was limited. Plasma clearance was rapid (within days), resulting in accumulation of vector in filtering organs, particularly the liver. Short-term clearance of the vector by the liver is likely followed by efficient degradation, for example, by liver Kupffer cells, because levels of vector DNA sequence in liver decline rapidly as well. In agreement with the rapid clearance from the circulation, spread of AAV1-LPL^{S447X} to distant organs such as liver did not result in transgene expression. Vector DNA was detected in both male and female gonads, but the levels declined over time to undetectable (low dose) or low (high dose) levels after 3 months (Rip *et al.* 2005). In LPL-/- cats, vector sequences were detected in testes en epididymides at 8-10 weeks after intramuscular AAV1-LPL^{S447X} delivery. However, the corresponding motile sperm fraction contained little (<10 copies) to no vector DNA sequence (Ross *et al.* 2006).

In the phase I/II clinical trial with AAV1-LPL^{S447X} vector clearance from the serum occurred at a rate of 1 to 2 logs per week and urine was free of vector sequences as early as one week after administration. Very low levels of maximally 25-28 vector copies/mg DNA could be detected in semen (Nierman *et al.* 2007).

2l. Information about mutation and recombination after *in vivo* administration

I Is the vector replication competent or replication deficient?

II What is the incidence of mutation and recombination of the parental virus?

III What is the incidence of mutation and recombination of the vector *in vitro/in vivo*

IV Information on complementation or missing functions in the vector by the wt virus

V Other treatments that may influence recombination/mutation

The vector is replication deficient and the likelihood of recombination is low, because it would require both co-infection with a wild-type AAV (to supply missing *rep* and *cap* genes) and a helper virus to complete the viral life cycle. There are no data available on the incidence of mutation and recombination of the parental virus. Even if such an unlikely event would occur and replication competent AAV vector would result in ongoing infection, this would clinically most likely result in a gastroenteritis or upper respiratory tract infection, although the total effect and in particularly possible toxicity depends largely on the transgene (see below).

2m. Information about possible toxicity (as a result of either the vector itself or the encoding transgenes)

AAV-1 was shown to be well tolerated in male and female C57Bl/6 mice (Rip *et al.* 2005). No deaths or significant changes in overall health or food intake were found, except for a reduced body weight gain in the high dose treatment group (-30% at the high dose of 10^{13} gc/kg). Marked overexpression of LPL in skeletal muscle has also been shown to result in reduced body weight gain in transgenic mice (Levak-Frank *et al.* 1995) and rabbits (Koike *et al.* 2004). No significant hematological or biochemical abnormalities were observed (Rip *et al.* 2005). Microscopically, a transient lymphoid hyperplasia was found in the spleen of animals in the high dose treatment group at days 7 and 28; a grade 1 myositis was found in all animals, both control and low-dose ($1x10^{11}$ gc/kg) treated, at day 7 as a result of the intramuscular injections, but histology was normalized by day 28 in all groups, except the high dose treatment group, which were found to display a grade 2 myositis at day 90 (Rip *et al.* 2005). Treatment of female CD1 mice with doses up to 10^{13} gc/kg at 4 weeks prior to mating, resulted in increasing concentrations of vector DNA in maternal tissues, but not in any of the fetuses and fetal death or abnormalities were not observed (Van Amersfoort *et al.* 2007; 2008). In addition, treatment of pregnant mice with AAV-LPL^{S447X} demonstrated the presence of vector DNA in the maternal, but not the fetal site of the placenta and absence of germline transmission (Van Amersfoort *et al.* 2007; 2008). Intramuscular treatment of LPL$^{-/-}$ mice with vector did not result in an increase in creatine phosphokinase (CPK), a marker of muscle injury, or local signs of toxicity due to the injection, the AAV vector or LPL expression (Ross *et al.* 2004). In the LPL$^{-/-}$ cat model, CPK levels did increase in response to AAV-LPL^{S447X}, with peak levels at week 3-4 and normalization of CPK to baseline levels at week 8 (Ross *et al.* 2006). Treatment with Cyclophosphamide could not prevent the increase in CPK levels, but in cats that did not generate an immune response, CPK levels were generally lower (Ross *et al.* 2006). In previous clinical trials, except for a minor discomfort during injection, there were no signs of hepato- or nephrotoxicity after injection and later during follow-up (Stroes *et al.* 2008). Muscle function tests and fat content were unaffected by AAV-LPL^{S447X} and only 1 patient developed a transient increase in serum CPK

levels at 4 weeks postinjection, coinciding with a loss of transgene expression and suggestive of T-cell mediated destruction of transduced muscle cells (Stroes *et al*. 2008; Mingozzi *et al*. 2009).

2n. Environmental shedding

There is a substantial amount of literature available suggesting that shedding of rAAV is dependent on the dose and route of administration, and that vector DNA can be detected for a number of weeks in serum, and early times i.e. day 1 post administration, in saliva, serum, urine and semen (Favre *et al*. 2001; Manno *et al*. 2006; Provost *et al*. 2005). Ideally, if positive DNA signals are observed, the samples should be followed up for infectious virus quantification. The data derived from non-clinical shedding studies and from early phase clinical studies can then be used to assess the likelihood of transmission and to justify the extent of viral shedding evaluation in subsequent trials. Since shedding of AAV, if any, is expected to come from respiratory secretions, stool, urine and semen, and therefore these sources will be monitored. However, after intramuscular injection, the highest risk of shedding is during initial injection and risks are expected to be very low thereafter (see above).

2o. Horizontal transmission

Horizontal transmission is highly unlikely to occur with the proposed safety precautions (see ERA, part A, step 4). In case of inadvertent transmission, immunity against AAV will rapidly clear the vector and no serious toxicity is expected from either the vector or the transgene (Rip *et al*. 2005).

2p. Safety back-up

I Is a suicide gene present?

II Are the vectors sensitive to anti-viral agents?

There is no suicide gene present in the vector construct and there are currently no treatment options for persons inadvertantly exposed to the vector.

Step 3: Information about production of the vector

3a. Which producer cell line(s) are used?

3b. Which viral functions are provided by the cell lines?

3c. Which quality control measures are used?

3d. Which criteria are used to reject a batch?

rAAV for use in gene therapy was previously produced in mammalian cell culture systems by providing DNA plasmids that contain the therapeutic gene flanked by the ITRs of AAV replication, genes for AAV replication and genes for virion or structural proteins. In addition, a plasmid containing adenoviral genes was provided to enhance the expression of the AAV genes and improve vector yield (Grimm *et al.* 2008). Nevertheless, in most mammalian cell culture systems, the number of AAV particles generated per cell is ~10^4 viral particles (Clark *et al.* 2002), whereas for a clinical study >10^{15} particles of rAAV may be required. Large scale production of clinical grade rAAV vector has proven difficult to achieve in mammalian cell culture systems, but have been proven feasible in the baculovirus insect cell system (Meghrous *et al.* 2005). Thus, to overcome production problems, the current replication incompetent rAAV-LPL^{S447X} vector is produced in a baculovirus system (Urabe *et al.* 2002). Baculovirus is produced using the Bac-to-Bac baculovirus expression system (Invitrogen). rBac-Cap is amplified by infecting 2x10^6 Sf9 (Spodoptera Frugiperda) cells and the supernatant containing the virus is recovered after 3 days. rAAV batches are produced using three recombinant baculoviruses (Urabe *et al.* 2002): the first baculovirus contains the construct for the LPL^{S447X} transgene, the second baculovirus harbors the AAV replication genes, Rep 78 and Rep 52, the third baculovirus harbors the AAVl capsid sequence. In comparison to the mammalian system, the rAAV construct produced in the baculovirus insect system has a significantly higher amount of VPl compared to the amount of VP2 in the capsid, resulting in virus particles with improved infectivity. AAV particles are purified by affinity-purification, using an immobilized monoclonal antibody against an AAV capsid protein. In view of the safety of viral vectors it is desirable to construct a viral vector unable to propagate after initial introduction into a cell. An AAV replicating in a mammalian cell typically has two ITR sequences. By using a chimeric ITR, ie a single ITR suffices for AAV propagation of a circular vector, increased safety is provided (US2003148506).

PART C: IMMUNITY-RELATED ISSUES

Step 1: What is the level of host immunity?

1a. Do(es) the patient(s) have a functional immune system: Is the patient immune-competent or (relatively) immune-deficient. If the patient is immune impaired, what is the extent of the immune deficiency?

I impaired innate immune system

II impaired adaptive/cellular immune system
- primary immune deficiency (PID)
- secondary immune deficiency (AIDS)
- induced transient immune suppression (chemo/ radiotherapy)

All patients are adults over 18 years of age and with a normal and functional immune system.

1b. Do(es) the patient(s) have a pre-existing condition (co-morbidity) affecting immunity, such as a mental or physical disability or for example diabetes?

I Due to the presence of any or more of risk factors

II Due to increased risk of exposure to a specific virus

Patients with a pre-existing condition other than LPL deficiency, affecting the general health and/or immune status are excluded from the trial. Some patients may have drug-controlled diabetes.

1c. Do(es) the patient(s) have proven pre-existing or cross-reacting immunity (presence of antibodies) against the viral vector or is it expected that the patient may have pre-existing or cross-reacting immunity against the viral vector?

I previous infection with the wt virus or a closely related virus

II previous vaccination with the wt virus or closely related virus

III previous treatment with this viral vector or a similar vector

IV previous contact with the transgene, eg enzyme replacement therapy

V confirmed presence of neutralizing or cross-reacting antibodies

In a recent study of the prevalence of IgG and neutralizing factors to AAV types 1, 2, 5, 6, 8 and 9 in the human population, it was shown that natural exposure to AAV resulted in the production of antibodies from all four IgG subclasses, with a predominant IgG1 response and very low IgG2, 3 and 4 responses. Prevalences of anti-AAV1 and 2 total IgG were highest (67and 72%, respectively) and followed by those of anti-AAV5 (40%), 6 (46%), 8 (38%), and 9 (47%) (Sylvie *et al.* 2010). There are currently no vaccinition programs against AAV and previous treatment with a gene therapy vectors is a factor to exclude patients from this trial. There is currently no enzyme replacement therapy available for the treament of LPL deficiency and in a recent clinical trial with AAV-LPL^{S447X}, 4 out of 8 subjects developed T-cell responses to capsid proteins and IgG3 anti-AAV-antibodies with a dose-dependent kinetics of appearance, but none of the subjects developed B- or T-cell responses to the LPL transgene product (Mingozzi *et al.* 2009).

1d. Is the patient a known or suspected carrier of a virus, similar or closely related to the viral strain, used for the vector (presence of viral nucleic acids)?

I Does the patient have a history of infection(s) with wt virus used as viral vector?

II Does the patient have a current infection or inflammation?

Patients with active infections, such as with adenoviral, herpes simplex virus or vaccinia, are excluded from the trial as these viruses are known helper viruses to AAV. Selecting AAV naïve subjects is currently not possible due to lack of (sensitivity of) available assays and also most humans are already exposed to wild-type AAV before the age 5 (Mingozzi and High, 2007; Sylvie *et al.* 2010). Prior to inclusion in the study, patients will be screened for the presence of pre-existing neutralizing anti-AAV-1 capsid antibodies.

Step 2: What type of immune modulation is used?

2a. What is the type of immune modulation(s) used? Stratify according to 4 risk categories:

I Systemic immune suppression with drugs resulting in total myeloablation, severe myelosuppression or T-cell depletion (in particular ATG or Alemtuzumab)

II Specific targeted immune modulation with agents blocking co-stimulation

III Steroids

IV Other

Combination treatment with Mycophenolate Mofetil, Cyclosporine A and methylprednisolone (category I)

2b. What is the dose (single or multiple) and duration of the treatment(s) (and in between treatments)?

Patients will be treated with Mycophenolate Mofetil per os at 2 g/day from day -3 till week 12; Cyclosporine A per os at 3 mg/kg/day from day -3 till week 12 (both risk cat-

egory I); and methylprednisolone, as a single intravenous bolus of 1 mg/kg bodyweight (risk category III). Together this protocol induces a systemic immune suppression that can be regarded moderately severe-severe.

2c. What is the anticipated (intended) level and duration of immune suppression achieved with this type of immune modulation? Ie, is the immune suppression intended to be transient or long-term.

These regimens are initially based on regimens used in the suppression of immune responses during kidney transplantation, but have been tested in several animal models as well. In clinical kidney transplantation the traditional or "conventional" immunosuppressive protocol generally consists of a calcineurin inhibitor (Cyclosporine A or Tacrolimus), an antimetabolite as adjunctive (Azathioprine or MMF), and prednisone (Yabu and Vicenti, 2009). Cyclosporine A (CsA) specifically and reversibly inhibits proliferation of T-lymphocytes, but does not affect hematopoiesis or disrupt the function of phagocytic cells of the innate immune system. In kidney transplant studies, these regimens were shown to offer an excellent long-term safety profile. A reasonable starting point for the design of a regimen to block the CD8+ T-cell response to AAV capsid is therefore to use regimens used in organ transplantation. However, whereas the goal in organ transplantation is to block T-cell responses to thousands of antigens on the donated organ, in AAV-mediated gene transfer, the goal is to block the T-cell response to the vector capsid, a single antigen that is only transiently present and is not actively expressed.

2d. What is the rationale behind the choice of immune modulation?

I Based on induced immune response against the vector

II Based on animal studies

III Other

In both mouse and cat models, induction of an immune response was observed after intramuscular injection and as a result efficacy of gene therapy was transient, because of an anti-hLPL neutralizing antibody immune response blunting LPL expression. In the cat model, the level and duration of efficacy were significantly improved with cyclophosphamide immunosuppression (Ross *et al.* 2006). LPL-/- cats on a commercial cat-food diet were treated with increasing doses of AAV-LPL^{S447X} up to 1x10^{12} gc/kg im and treated with 100-200 mg/m^2/wk Cyclophosphamide po. Immune suppression with cyclophosphamide improved short-term efficacy after administration of a high vector dose, and prevented formation of anti-LPL antibodies in 2 out of 3 cats treated with the lowest vector dose (1x10^{11} gc/kg) (Ross *et al.* 2006). In other studies using intramuscular delivery of AAV vectors for the treatment of hemophilia B in dogs, it was shown that immune suppression with Cyclophosphamide started before but not after AAV1-fIX delivery could prevent the formation of anti-fIX antibodies (Arruda *et al.* 2004).

Step 3: What is the effect of this particular immune modulation regimen on the patient's risk of infection?

3a. Is the immune modulation used associated with an increased risk for primary infection?

With the conventional kidney transplant immune suppression protocols (Yabu and Vicenti, 2009), consisting of a CNI (cyclosporine or tacrolimus), an antimetabolite (azathioprine or MMF), and prednisone, which is the basis of the immune suppression suggested in this clinical trial, susceptibility for and reactivation of different viral infections are following a certain time pattern. Both CsA and MMF are associated with an increased risk for Herpes infection, in particular CMV. Short term treatment with methylprednisolon is not clearly associated with any type of infection, but it may increase the risk for primary infections, when combined with CsA and MMF. The effects of this type of immune modulation are not long-lasting, such as with ATG, and a rapid recovery of immune competence is expected after withdrawal of the treatment. As with monitoring of organ transplant recipients, prevention of viral infections is necessary and upon signs of viral infection, immune suppressive agents will be lowered or stopped.

3b. Is the immune modulation used associated with reactivation of latent viral infection?

During the first 1-2 months, patients with a history of HSV may have a reactivation, followed by infection (2-4 months) with other herpes viruses such as CMV, EBV, VZV, HHV8, Parvovirus B19 and measles. Community-acquired infections such as influenza, RSV, and adenovirus may appear at any time (Kotton and Fishman, 2005). Here, the intended use of CsA and MMF is up to 12 weeks after gene transfer. This may be sufficient to allow tolerance to the transgene, while preventing long term infectious complications.

Step 4: Is the patient subject to other treatments that may affect the immune modulation facilitated gene therapy treatment?

4a. Which treatments are (possibly) used, at what dose and duration?

4b. Do these treatments in any way affect the functioning, biokinetics and half-life of the immune modulatory agents?

4c. Do these treatments affect in any way the patient's immune system?

4d. Do these treatments influence the viral vector, by interfering with biodistribution, persistence, viral life cycle, recombination and shedding?
The patients are not subject to any other treatment protocols other than a fat-restricted diet.

Step 5: Does the immune modulation in any way affect viral vector biodistribution and persistence?

5a. What are the relevant animal/clinical studies?
See question 5b.

5b. How is biodistribution of the vector affected by immune modulation?
Cyclophosphamide had been shown previously to delay the onset of the antibody responses to AAV-1-mediated transgene expression of factor IX in a dog model of hemophilia B (Arruda *et al.* 2004). In a LPL-/- cat model, the effects of different doses of cyclophosphamide ranging from 100-200 mg/m^2 per week were tested on the immune response against AAV-LPL^{S447X} and were shown to significantly improve both the level and duration of efficacy (Ross *et al.* 2006). AAV-LPL^{S447X} gene therapy in cats demonstrated prolonged resolution of visible plasma lipemia and a 96% reduction of plasma TGs lasting over 8 weeks when combined with immunosuppressive treatment. However, after cessation of the immune suppressive treatment at 8 weeks of treatment, an anti-human LPL immune response occurred, resulting in loss of efficacy and an increase in plasma CPK levels. In addition, after local im injection of the AAV vector and immune suppressive treatment, expression and activity of the LPL protein was found only in treated muscles and not in untreated muscle biopsies or liver samples (Ross *et al.* 2006), indicating that rAAV1-mediated LPL^{S447X} expression remains localized within the injected muscle tissue as reported previously in non-immune suppressed animals (Ross *et al.* 2004). In a non-human primate model of hemophilia B, the effects of combinations of other immune suppressive agents on the formation of anti-AAV2/8 antibodies and transgene expression of fIX were tested (Mingozzi *et al.* 2007). Regimens as used in renal transplant patients, including an antimetabolite, such as Mycofenolate Mofetil (MMF) and an mTOR inhibitor, such as sirolimus, have been shown to have a long term safety profile and were used to prevent an immune response against AAV and human fIX in rhesus monkeys (Mingozzi *et al.* 2007). Monkeys received 25mg/kg MMF twice daily po from 1 week before gene therapy to 10 weeks thereafter and Sirolimus, which is often used in the clinics in patients unable to take Cyclosporin treatment, at a dose of 4 mg/kg/d po from day +1 to day +7 and 2 mg/kg/d thereafter up to 10 weeks after gene therapy. Such a regimen of transient immune suppression did not substantially alter either transduction efficiency or vector biodistribution of AAV-2-mediated liver-directed gene therapy (Mingozzi *et al.* 2007). Previous studies in non-human primates treated with AAV-8-fIX demonstrated that a regimen composed of MMF and tacrolimus (FK-506) does not affect the liver transduction efficiency or biodistribution of the vector (Jiang *et al.* 2006).

5c. How was biodistribution and persistence of the vector measured?
In the non-human primate model, as described above, using an immune suppression regimen consisting of MMF and sirolimus, biodistribution and vector persistence were measured using real-time quantitative PCR to determine vector copy number in liver tissue at 8 weeks and of various other tissues (spleen, testis, kidney, thymus, heart, skeletal muscle, diaphragm, lung, inguinal lymphnodes and different liver lobes) at 40 weeks. No substantial differences were detected in vector biodistribution after direct injection into hepatic artery of AAV2-fIX vector (Mingozzi *et al.* 2007). In the LPL-/- cat study using AAV1-LPL^{S447X} im, treatment with Cyclophosphamide did not result in changes in the biodistribution of LPL activity or protein expression as measured with an ELISA (Ross *et al.* 2006). Other models using AAV2-cfIX in canine hemophilia missense and nonsense models in combination with Cyclophosphamide only looked at formation of antibodies against the transgene and the vector (Herzog *et al.* 2001; 2002) and not at biodistribution or vector persistence. The study of AAV-8-fIX in non-human primates, transiently treated with MMF and FK-506, used realtime QPCR at 6 months after intrahepatic vector injection to assess gDNAs from various tissues, including heart, kidney, liver, testis, lung, spleen and thymus (Jiang *et al.* 2006). The limit of detection was 8x10^{-5} vector copies/diploid genome.

5d. How long were biodistribution and persistence measured and up till what time were samples found to contain evidence of viral presence?
This strongly depended on the type of study, as described above at 5b and 5c.

Step 6: Does immune modulation affect the likelihood of recombination or reassortment of the viral vector?

6a. What are the relevant studies?
None.

6b. How are recombination or reassortment affected?
Immune modulation does not appear to affect the likelihood of recombination or reassortment of the AAV1-LPL^{S447X} vector. The risks for recombination or reassortment of AAV-1 are very low under study conditions where no immune suppression is used and although only limited data are available on the use of immune modulation therapy to prevent an immune response against an AAV vector or transgene, thus far no data have been published that indicate an increased risk for recombination or reassortment in cases where immune modulation is used.

Step 7: Does immune modulation affect the risk for shedding and transmission of the viral vector?

7a. What are the relevant pre(clinical) studies?
I How was shedding measured?
From which sides is shedding measured?
II How often and how long was shedding measured?
At which time points is shedding measured and up till what time.
III How is secondary/tertiary transmission measured?
Which method is used.
IV Were any negative effects of secondary/tertiary transmission documented?

A phase I/II clinical trial of AAV-LPL (1 or $3x10^{11}$ gc/kg) assessed the excretion and shedding of a previous AAV-LPL vector batch, which was produced in HEK293 cells and injected im (Nierman *et al.* 2007). With a sensitive quantitative PCR, vector sequences could be transiently detected in serum, saliva, urine, semen, and muscle biopsies. The highest vector concentrations were detected in the serum, with a rapid clearance by 1–2 logs/week. Urine was free of vector sequences 1 week after treatment in the first dose cohort. Very low levels (at maximum 25–58 copies/mg DNA) were detected in the semen for short periods of time, making the risk for germline transmission extremely low. Persistent presence of high levels of vector sequences was only detected in injected muscle and leakage of vector from the injection site was limited (Nierman *et al.* 2007). No clinical data are presently available on shedding during AAV-LPL treatment in combination with immune suppression and only limited animal studies in healthy animals or disease models have been performed, as described above, using similar types of vectors, but different types of immune modulation. Nevertheless, it is expected that the effects of immune modulation will be minimal since 1) the AAV vectors do not replicate; 2) the vectors are continually diluted from the point of administration to potential sites of shedding; 3) shedding is limited by cell barriers even if the immune system is relatively suppressed.

Vector shedding can be measured by using Q-PCR techniques or bioassays, such as the replication or infectivity assay, and although Q-PCR is more sensitive, the infectivity assay is more informative about the possible implications of viral shedding. The Q-PCR should be designed with a set of primers able to distinguish between the mutated gene and the transgene as well as differentiate the vector from wild type AAV. There are no data available on secondary or tertiary transmission, but even in case of shedding and transmission, the AAV vector does not propagate outside of cells and as discussed before, the risks for recombination or reassortment of AAV vectors are very low and require the presence of a wild-type AAV and a helper virus; wild type AAV itself is not known to be pathogenic and the transgene is not associated with any negative effects in the normal population.

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A possible remark from the governmental institutions addressing this fictional trial could have been the following:

The immune suppressive effects of CsA in animal models to prevent an immune reaction against gene therapy vectors is minimal to low, see Table II. Furthermore, this regimen was chosen based on previous experience with the use during clinical kidney transplantation. In a review paper on the topic by Yabu and Vicenti in 2009, however, it was stated that "*compared with cyclosporine, tacrolimus has been shown to have a more favorable effect on hypertension and lipid profiles and is often preferred over cyclosporine in recipients with difficult-to-control hypertension or severe dyslipidemia*". In addition, one of the known side effects of CsA is pancreatitis and hyperlipidemia and additional use of methylprednisolon is known to increase blood levels of CsA (Farmacotherapeutisch Kompas 2010, http://www.fk.cvz.nl). In particular in a patient population with hyperlipidemia and an increased risk of lethal pancreatitis, this choice of immune suppressive regimen may need a better substantiation. For example, if other immune suppressive regimens are not possible due to other reasons, these should be mentioned in the applications. A rational treatment choice could have been the substitution of CsA for Tacrolimus or an altogether different type of immune suppression, such as Cyclophosphamide, which was effective in most animal models and has also been tested in preclinical trials of AAV-LPL.

3.4 ERA sample animal study

This section consists of an example of a fictional ERA with a replication-deficient adenoviral vector, which is used in animal studies using immune suppressed or immune deficient animals. Only part C of the ERA, concerning immunity-related issues, is discussed here. The example is based on the use of an immune competent hemophilia B mouse model, using a second generation E1/E2a/E3-deleted hAd5 vector with a CMV enhancer/promoter and a therapeutic transgene (hfIX) and immune modulation with cyclophosphamide. The vector is produced in the 293 helper cell line, which harbors no sequence overlap with the vector, preventing homologous recombination and formation of replication competent Ad (RCA). Although it is possible that a revertant E1 Ad (REA) or that helper-dependent E1-positive particles (HDEP) are formed, the additional absence of E2a prevents the formation of replication compentent virus. As homologous recombination with wild-type Ad could occur after *in*

vivo gene therapy, resulting in replication-competent virus, all animals should be free of wild-type Ad.

Adenoviruses are infectious for human beings and often cause mild respiratory illness, eye infections or gastroenteritis. Although hAd5 only causes mild respiratory infections in healthy humans, it may cause fatal kidney or lung infections in immune compromised patients. As rare cases of severe disease may occur, its use as a genetic vector requires the use of adequate containment equipment and practices. Biosafety containment level 2 for animal facilities using micro-organisms (DM-II) is appropriate for standard use of many adenoviral constructs in rats or mice (CGM/000330-01) and are considered sufficient to prevent transmission of aerosol Ad particles in which case some additional measures are taken (i.e. use of filtertop cages, wearing gloves during procedures, work in safety cabinet class II in case cage is opened with a contaminated filtertop). Particular care should be given to vectors containing genes that make products that may be similar to products made by the deleted adenovirus genes, even though this does not affect the DM-II containment level: both replication-deficient and replication-competent adenoviral vectors are contained at this level.

Two scenarios are envisaged by which immune modulation may influence the risk assessment in contained use applications: 1) the presence of an immune modulatory transgene might change pathogenicity or virulence of the vector in comparison to the wild-type virus (may affect containment level); 2) the use of a replication-competent or deficient virus in an immune suppressed or immune compromised animal could prolong the halflife of the vector, vector distribution and viral shedding and hence, the risk assessment for instance in case experiments need to be carried out at lower containment level. The latter is discussed in this preclinical ERA example.

PART C: IMMUNITY-RELATED ISSUES

Step 1: Information about host immunity

1a. Does the animal model have a functional immune system

Yes. The animal model used here is the hemophilia B C57Bl6/J/129 mouse, which has a large deletion of fIX and is fully immune competent.

1b. Does the animal model have a condition affecting immunity

No.

1c. Does the animal model have pre-existing or cross-reacting immunity

No.

1d. Are the animals carriers of a related virus

No. All animals are housed under strict conditions as prescribed by the Federation of European Laboratory Animal Science Associations (FELASA). This ensures the absence of murine adenoviruses in the mice and requires regular screening. Even if the animals would be carriers of a murine Ad, the risks of recombination between the used hAd5-FIX vector and the wt murine Ad would be very low, due to the minimal sequence homology between murine and human Ad.

Step 2: Information about immune modulation

2a. What is the type of immune modulation(s) used

Cyclophosphamide (cytoxan, CY)

2b. What is the dose and duration of the treatment(s)

CY is administered in 200 ml saline at doses of 20 or 50 mg/kg by intraperitoneal (IP) injection at the day of vector administration (day 0) and biweekly thereafter up to week 6.

2c. What is the level and duration of immune suppression

See 2b.

2d. Explain the choice of immune modulation

CY is a commonly used immune suppressant, known to inhibit B-cells and CD4+ and CD8+ T-cells and has been used for this reason in multiple animal models (Dai *et al.* 1995, Jooss *et al.* 1996, Fields *et al.* 2001).

Step 3: Effect of immune modulation on the risk of infection

3a. Increased risk for primary infection

The animals might be more susceptible to primary infections due to the immune suppressive treatment, but since the animals are kept in filtertop cages at DM-II level and handled under strictly controlled FELASA conditions, the risk for primary infections is considered negligible. These conditions have previously been sufficient to sustain animals receiving lethal doses of radiation and immune deficient mice without the complications of infections. If the animals require transfer from their cages to a lower level of containment (D-I area), for example for imaging procedures, additional precautions need to be taken to prevent infection. General precautions are taken to prevent transfer of possible infectious viral particles from

the animals to the researchers and vice versa, to prevent primary infection of mice by laboratory personnel and contamination of the laboratory and imaging device. Additional measures may include the use of sterile transport filtertop cages for the mice, and masks, gloves and laboratory glasses for researchers, and sedation of animals. The imaging area is cleaned with 1% SDS and 70% ethanol, to kill wt virus particles before use and again after animal imaging to prevent transfer of possible viable infectious vector particles.

3b. Increased risk for reactivation of latent infection
All animals are specific-pathogen free (SPF) animals and are born and kept under controlled conditions (FELASA). The risks for reactivation of latent infections is therefore considered negligible.

Step 4: Information about other treatments
4a. Which other treatments are used, at what dose and duration
Mice will receive ciprofloxacin in their drinking water to prevent bacterial infections.

4b. Do these treatments affect the immune modulatory agents
Yes. It was shown in rats (Xie *et al.* 2003) and humans (Afsharian *et al.* 2005) that ciprofloxacin alters the pharmacokinetics of CY. CY is a prodrug, which requires hepatic biotransformation and cytochrome P450 to exert its cytotoxic effect. Ciprofloxacin (Cipro) is a fluoroquinolone agent, which has gained widespread use in the treatment of a broad range of infections, but has been shown to inhibit Cytochrome P450 enzymes. It was shown that Cipro affects CY metabolism, when used at high doses, but not at lower doses. Therefore, in this study two different doses of CY, ie 20 mg/mg and 50 mg/kg will be tested.

4c. Do these treatments affect the animal's immune system
A rare side effect of Cipro is that in some cases Cipro may result in pancytopenia and bone marrow depression (0.01-0.1%) in human beings. However, in our experience, Cipro has never been associated with any form of cytopenia in mice.

4d. Do these treatments influence vector kinetics
No. Cipro affects DNA synthesis by inhibition of bacterial DNA-gyrase. This is not expected to influence vector kinetics. In addition, drugs affecting the cytochrome P450 system are not known to affect vector kinetics. However, systemic Ad5 administration has been shown in a rat model to negatively affect at least two cytochrome P450 enzymes (Callahan *et al.* 2005) and therefore may affect CY levels.

Step 5: Effect immune modulation on vector distribution and persistence
5a. What are the relevant animal/clinical studies
Comparable animal studies were done in normal mice treated with different types of immune suppressive drugs, including a study using CY and Cyclosporin A (CsA) with an $1x10^9$ pfu Ad5-CMV-hfIX vector im (Dai *et al.* 1995). They showed that CsA alone had no effect while CY with or without CsA allowed prolonged transgene expression. A second study was done in normal mice injected with $5x10^8$ pfu Ad5-CMV-lacZ iv. Here CY prolonged transgene expression (Jooss *et al.* 1996). A third study was done in hemophilia B mice. Here $1x10^{11}$ vg of AAV-CMV-mfIX were injected iv. Different immune suppressive drugs, including CY, FK506, CsA, anti-CD40L and CTLA4-Ig were used (Fields *et al.* 2001). Here, it was shown that anti-CD40L has no effect on transgene expression, FK506 resulted in short-term partial correction of the phenotype, FK506 was affective as long as the drug was administered, CsA resulted in renal toxicity and death of animals and CY resulted in long-term systemic expression of fIX (Fields *et al.* 2001).

5b. Does immune modulation after vector biodistribution
There are no data available yet, but the purpose of this study is to see if immune modulation with CY will affect vector biodistribution and vector persistence, as well as prolonged systemic transgene expression.

5c. How are biodistribution and persistence measured
By using real-time quantitative PCR to determine vector copy number in liver tissue at 8 weeks and of various other tissues (spleen, testis, kidney, thymus, heart, skeletal muscle, diaphragm, lung, inguinal lymphnodes and different liver lobes) at 6 months and 12 months.

5d. How long is biodistribution/persistence measured
See 5c.

Step 6: Effect of immune modulation on recombination
6a. What are the relevant studies
There is no relevant study and the effect of immune modulation on recombination is currently unknown, but

will be a secondary readout in this study.

6b. How are recombination or reassortment affected
There are no available data. Recombination and formation of replication competent Ad (RCA) in this system can occur during the production process by homologous recombination and complementation of missing functions (E1) or after *in vivo* administration after recombination with wt Ad. The use of a second generation hAd5 with multiple deletions makes recombination during production highly unlikely, and the presence of RCA in the virus batches is an exclusion factor for the use of that virus batch. *In vivo* immune modulation however, does not affect vector production. Prolonged presence of hAd5 infectious virus particles due to the use of CY may increase the risk for recombination. However, animals used in this study are free of murine Ad and even in case of infection with murine Ad, the risk of recombination between a wt murine Ad and hAd5 is low due to minimal sequence homology. Thus, a recombination event resulting in the formation of RCA appears highly unlikely, even in case of prolonged immune suppression. The most likely scenario resulting in RCA would be co-infection of the mice by a wt human Ad derived from research or laboratory personnel. The proposed housing and working conditions (DM-II) and barrier safety measures, however, should minimize, if not completely prevent, these risks.

Step 7: Effect of immune modulation on risk for shedding and transmission
7a. What are the relevant pre(clinical) studies
One of the endpoints in this study will be whether iv administration of hAd5-hFIX and treatment with CY will result in (increased) shedding and possible transmission. For this reason, plasma, anal swaps, and mouse droppings will be tested for shedding of the viral vector.

Conclusions
Little is known about biodistribution, recombination and shedding of viral vectors in immune compromised or immune suppressed animals. It is anticipated that immune modulation or lack of an optimal immune system will allow for longer persistence of infectious viral particles in the animals. Studies as the one proposed above should be able to help answering these gaps in current knowledge. In this fictional preclinical ERA, we propose the use of a second generation E1/E2a/E3 deleted adenoviral vector based on human serotype 5 in a murine immune competent animal model of hemophilia using short term Cyclophosphamide to prevent an immune response against the viral vector and/or transgene. Although in this model prolonged presence of the vector is anticipated, possibly affecting biodistribution and shedding, it seems highly unlikely that with the currently required DM-II housing and safety procedures, infective RCA will occur due to sequence differences between murine and human Ad. Transfer of the animals after *in vivo* gene therapy with a replication-deficient hAd5 to D-I areas, for example for imaging purposes (eg MRI or bioluminescence) may be possible under certain restricted conditions (e.g. as described under step 3a). Other studies using hAd5 in *in vivo* models as described in COGEM advices CGM/031031-06 (hAd5 in pigs) and CGM/021216-03 (Ad in apes), but without the use of immune modulation, require the use of a specific test to demonstrate absence of shedding of infectious viral particles in serum, urine and faeces and/or RCA for at least 24 hours (pigs) and 7 days after gene therapy (apes). Pigs can be further housed under D-I conditions 3 days after *in vivo* gene therapy (CGM/031031-06), whereas apes can be housed under D-1 conditions 7 days after *in vivo* gene therapy when absence of shedding in feces has been confirmed with a validated PCR test for 7 consecutive days (CGM020513-03, CGM/021216-03). These requests are based on the relatively low level of sequence homology between porcine and human Ad (5-20%) and the high level of homology between simian and human Ad, as well as the fact that apes are possible carriers of wt human Ad and susceptible to primary hAd infections. In the case of immune modulation in the murine model, absence of shedding for 3 consecutive days with specific tests (eg validated PCR) in addition to the use of personal barrier restrictions (mouth masks, gloves, laboratory glasses) and cleaning measures (1% SDS and 70% ethanol) of used surface areas outside of the DM-II containment areas would result in a negligible increase in environmental risk.

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4 CONCLUSIONS AND RECOMMENDATIONS

4.1 Introduction

Host immune responses play a major role in the clearance from viral infections from the body. The induction of these immune responses are now being used to our advantage in the treatment and specific recognition of certain cancers, whereas for the treatment of monogenetic diseases, the same (acquired) host immune responses play a crucial role as a determinant of long-term expression and could hamper clinical efficacy. This study was initiated by the Netherlands National Institute for Public Health and the Environment (RIVM) to assess the possible environmental risks associated with the use of immune modulation in combination with gene therapy and the resulting report contains an overview of existing data. This chapter will give general conclusions and recommendations for the current policies in The Netherlands.

4.2 General conclusions

This project has been very timely: Just recently the first and very relevant review papers and a book on this topic of have been published (Arruda, Favaro and Finn – Strategies to modulate immune response: a new frontier for gene therapy, Mol Ther 2009; Nayak and Herzog – Immune responses to viral vectors, Gene Ther 2010 and Gene Therapy Immunology, Wiley-Blackwell 2009, edited by R. Herzog). From these publications as well as this report, it becomes clear that similar as in the transplantion field, the gene therapy field is learning how to circumvent, manipulate or suppress unwanted immune responses. New developments include vector engineering such as capsid engineering, miRNA-regulated expression cassettes; optimization of delivery techniques, administration to immune-privileged sites, taking advantage of organ-specific immune responses[171]. In addition, experience from the organ transplant field can be used as a first reference to use immune suppression in gene transfer protocols (reviewed in Appendix B). Still, the specific immune suppressive treatment (combination of drugs, period of time) needed for gene therapy purposes will be depending on the vector, disease, target tissue and the therapeutic outcome.

Please note that more extensive and in depth information, including separate conclusions on the different subtopics can be found in appendices below: immunogenicity of currently used viral vectors (Appendix A); all relevant non-gene therapy, clinical studies from related fields, including (stem cell) transplantation to assess the risk of co-infections with wild type viruses (Appendix B); vaccination studies with live attenuated viruses to assess the risks for shedding and transmission (Appendix C); and all preclinical gene therapy studies using any type of immune modulation (Table II). Furthermore, practical suggestions on extension of current ERA forms have been provided in chapter 3, as well as samples for a clinical and animal study. Performing this exercise makes one realise once again the emerging importance of documentation and accessibility of clinical data to other/relevant parties, not only for the ERA but also for the benefit of individual patients.

4.2.1 Indications for immune modulation

Immune modulation in a clinical gene therapy setting may occur for multiple reasons:

I To balance the immune response against the vector, transgene and transgenic protein to achieve a maximal therapeutic effect and tolerance against the exogenous antigen. This requires a relative modest, short-term immune modulation, which changes the balance between effector T-cells and regulatory T-cells in favor of the latter. Viral vectors may be replication deficient or and replication competent and carry a therapeutic transgene.

II To treat a malignancy. This requires the use of combinations of cytotoxic agents, which may directly affect innate and adaptive immunity, but usually maintains some level of pre-existing immunity. Oncolytic viral vectors specifically target and replicate in malignant cells and are used with or without a transgene, which may require local activation.

III To create space in the bone marrow for engraftment of gene-corrected hematopoietic cells. Myeloablative treatment is used, resulting in the long-term absence of a functional adaptive immune system and possible loss of pre-existing immunity to common viruses. Transductions of cells are done *in vitro* rather than *in vivo*.

4.2.2 Risks of immune modulation

The most common risk of immune suppression is the increased susceptibility to opportunistic infections or reactivation of latent infections. The type of viral infection that occurs, depends on the specific combinations of immune suppression applied (see Table III) and the duration (and depth) of the treatment. In general, it is expected that this will render no difficulties in view of the risk assessment related to viral recombination events, since these will not occur between the virus species observed and viral vector applied.

Conclusions in general relating to risk assessment of immune modulation in clinical gene therapy studies: (1) choice of immune modulation during (pre)treatment is most relevant for the risk assessment relating to the individual patient; (2) modification of the viral vector system resulting in immune escape could be most relevant for risk assessment of the environment.

4.2.3 Relevance regulatory affairs

Finally, this project is also very timely since the first clinical trial has now been started (in Canada for LPL) and the second is about to start (in USA for Hemophilia). The first clinical gene therapy study including immune modulation in The Netherlands can be expected in the upcoming year(s). Adaptation and further implementation of findings obtained in this study in regulatory processing will therefore be availabe in time and delays can be prevented.

4.3 Recommendations

Preclinical animal studies and readout systems
- Application forms animal experiments extension with explicit questions on anticipating (environmental) risk assesment and obtaining relevant data

Clinical studies & Immune modulation
- New area of expertise with further ever emerging need of documentation and accessibility of data available to other parties

Precautionary measures
- Overview of education and training of hospital personnel involved during application of gene therapy and effects of precautionary measures used to prevent shedding and transmission

Regulatory aspects and risk assessment
- Proposal of extended version of current ERA for preclinical studies
- Proposal of extended version current ERA for clinical studies
- Proposal future project

4.3.1 Preclinical animal studies and readout systems

Application forms animal experiments extension with explicit questions on anticipating (environmental) risk assesment and obtaining relevant data.

As emphasized in the summary above and throughout the report, the effects of or the risks associated with immune modulation during gene therapy cannot be easily translated from the current animal studies to the clinic. The immune functions of the animal models used and their responses to viral vectors and immune modulation do not *per se* reflect the situation in patients. It is recommended that gene therapy animal models using immune modulation, in addition to transduction efficiency of the target tissue, transgene expression or anti-tumor effect also measure secondary outcomes such as changes in biodistribution and persistence, which are more relevant for prediction of clinical and environmental risks. Awareness of the researchers to include these kind of more clinically relevant readouts in addition to their primary readouts could be increased by additional questions in the application forms for animal experimental studies, addressed by the animal experimental committee (DEC). This could result in a better implementation of the principles of the three R's, ie replacement, reduction and refinement, as introduced by Russell and Burch in the nineteen fifties[172]. By making a small adjustment/addition to ongoing animal studies, the distribution and presence of viral vectors and genomes in different organs could be easily fully documented after necropsy, without increasing the number of animals needed and without increasing the discomfort of the animals themselves, but with an enormous gain of knowledge about vector persistence and distribution and a more clear picture of possible clinically relevant risks. In addition, preclinical studies in animal models measuring transfer of vector strain virus might be of additional value here and an animal study, which would include the highest possible immune suppression in combination with a replication competent viral vector would be a reflection of the ultimate "worst case scenario" and could probably best predict the possible risks, including recombination, shedding and transmission. However, these types of studies are unlikely to be initiated by separate research groups and might require the specific request from governemental policy institutes. Nevertheless, the importance of these studies is clear and may result in a much better understanding of how to translate shedding and transmission data from preclinical gene therapy and vaccination studies in animals to the human situation.

4.3.2 Clinical studies & Immune modulation

New area of expertise with further ever emerging need of documentation and accessibility of data available to other parties.

Importantly, the choice of a particular immune suppressive regimen in the clinic is currently based on clinical experience available from organ transplantation protocols, rather than whether this protocol would be truly

advantageous for this particular patient population and this specific viral vector system. The considerations which may play a role in the choice of immune modulation for long-term immune suppressive treatment to prevent organ rejection may be quite different from the ones in gene therapy, where short-term, low dose immune suppression may suffice. The choice of immune modulation therefore, should be strongly substantiated by either data from animal studies, other clinical studies, if available. In fact the use of immune modulation in patient populations, in which immune modulation has not been used or indicated before, should be a new area of expertise. As the immune suppression is relatively mild and intended for short-term use, patient responses, the risk of infectious complications and side-effects may be different. Unintended or insufficient responses could be monitored and a transparent, accessible databank for other researchers with patient results, eg shedding, transmission, education, etc after treatment with gene therapy with or without immune modulation could be maintained.

4.3.3 Precautionary measures

Overview of education and training of hospital personnel involved during application of gene therapy and effects of precautionary measures used to prevent shedding and transmission.

From a safety point of view, the most important issue is which precautionary measures are taken by the personnel and bystanders, during the processing of the vectors, the transduction and handling procedures, the transport to the patient and during final administration. The risks of shedding and transmission are not negligible during these procedures and more likely higher than after biodistribution and persistence in a patient, even when this patient is treated with immune modulation. Independent of whether shedding may occur during administration or later after persistence in the patient, prevention of transmission should be an important focus. In the vaccination studies is was shown that despite education of bystanders a certain level of vaccine strain transmission was not uncommon (Appendix C). In addition, not only shedding and transmission was measured but also survival of the vaccine strains outside the vaccinee and in the surroundings of the vaccinee were measured. Although there are general recommendations on how to act in case of spilling or during administration of various viral vectors, there is no literature available, which describes whether these recommendations are followed and whether or not they are effectively used. A database of clinical gene therapy protocols and shedding/transmission data is warranted. In addition, training and education programs for laboratory and hospital personnel, as well as for house hold members and other care takers may result in an increased awareness and a decreased risk of inadvertent transmission.

4.3.4 Regulatory aspects and risk assessment

• *Proposal of extended version current ERA for clinical studies*

We propose an extended version of a previously proposed ERA for clinical studies[75], which can be used when starting a clinical gene therapy trial with immune modulation and can also be relevant in making decisions for screening and monitoring patients and their environment, including health-care personnel and house hold members, during the trial. The (extension of the) ERA consists of three parts, focusing on the patients involved, vector characteristics and effects of immune modulation (chapter 3.3 ERA sample clinical trial).

• *Proposal of extended version of current ERA for preclinical studies*

We propose an extention of the current ERA for preclinical studies. This can be helpful when assessing risks in contained use applications (chapter 3.4 ERA sample animal study).

• *Proposal future project*

Highest risks remain during preparation and moment of administration of vector underlining the importance of knowledge, training and education. We propose a special project to assess the risks of vector shedding and transmission before, during and shortly after administration. This project should include data on which precautionary measures are used in clininal gene therapy trials with different viral vectors; should assess actual compliance with these measures; should contain information on how education of hospital personnel and family members are instructed to prevent transmission, and should if possible contain recommendations to improve all of the above. We also propose the establishment of an open and interactive database for monitoring the clinical aspects of gene therapy trials with or without immune modulation and access to standardized protocols on how to prepare patients for gene therapy trials to increase safety and allow more rapid adaptation of these protocols in response to new clinical findings. New or unexpected findings are now often only presented in medical literature and the time between important observations and publication is

protracted. Also negative findings, difficult to publish, could be discussed here.

4.4 Future expectations and developments

Further developments in the field of immune modulation in gene therapy will be in the area of vector modifications and should be closely monitored. From first generation to second generation and last generation vectors, a decrease in immunogenicity has been pursued and often achieved through relatively easy modifications, such as the use of different serotypes and a decrease in expression of viral genes. Futher reduction of immunogenicity is expected for the newest generations of vectors, which make use of stealth technology, restricted/regulated expression (microRNA and Tet-On tetracycline regulatable systems) and induction of tolerance. These and other new strategies to prevent immune responses for long-term gene therapy will eventually make the concomitant use of immune modulation less essential. Nevertheless, the use of immune modulation is likely to remain an important treatment strategy for diseases that require multiple gene therapy procedures. However, despite these exiting new developments todays gene therapy regimens still require the use of short-term, low-level immune modulation. Some decades of experience with immune suppressive agents in transplantation medicine has lead to the development of specific treatment regimens using different combinations of drugs. These have been shown to decrease dose-related side effects, while maximizing immune suppressive effects. Preclinical animal models using these types of regimens have been used with variable degrees of success and new clinical studies in gene therapy trials will have to show, which regimens should be preferred. The relative low frequency of these types of trials require open access to clinical data and an extensive sharing and exchanging of information for the benefit of the patients and for an optimal assessment of potential environmental risks.

APPENDIX A IMMUNE RESPONSE AGAINST VECTORS

Antigen presenting cells (APCs), such as macrophages (MΦ) and dendritic cells (DC), recognize pathogen-associated molecular patterns (PAMPs) with pattern recognition receptors (PRR), such as membrane-bound Toll-like receptors (TLRs) and intracytoplasmic RNA helicases (RIG-1, Mda-5). Recognition of viral RNA or DNA sequences triggers downstream signaling pathways, leading to induction of type I interferons (IFNα and β).

PPR	localization	pattern
TLR3	endosomal, cell surface	dsRNA, polyI:C
TLR7/8	endosomal	ssRNA, RNA homologs
TLR9	endosomal	CpG-DNA, CpG-ODN
RIG-1/Mda-5	intracyto-plasmic	viral RNA

The innate immunesystem is phylogenetically conserved and present in almost all multicellular organisms[173]. The functional components of the innate system are comprised of neutrophils, the mononuclear phagocytic cells such as blood monocytes and tissue macrophages, and natural killer cells as the main source of soluble factors, including complement, antimicrobial agents and inflammatory cytokines. Professional antigen-presenting cells, such as macrophages (MΦ) and dendritic cells (DCs) are present in various tissues throughout the body in an immature state[174]. Immature DCs patrol the tissues and sample their environment by pinocytosis of soluble material or phagocytosis of particles. It is the first line of defense upon entry of pathogens into the body and it recognizes conserved microbial structures, known as pathogen-associated molecular patterns (PAMPs) with germline coded pattern recognition receptors (PRR)[175]. The Toll-like receptors (TLRs) are membrane-bound PRRs and recognize a broad spectrum of microbial components, including viral vectors. RNA helicases are cytoplasmic sensors of viral infections and detect RNA. Recognition of viral RNA or DNA sequences by TLRs and RNA helicases triggers the activation of signaling pathways and induction of transcription of type I interferons (IFN-α and IFN-β), which are the principal mediators of the innate immune response to viruses, as well as other pro-inflammatory chemokines and cytokines which activate the adaptive immune response. TLR7/8 (present in mouse/man, respectively) and TLR9 are present in endosomes and recognize viral nucleic acids. TRL7/8 recognize ssRNA and ribonucleic acid homologs, such as R-848 (resquimod), gardiquimod and imiquimod; TLR9 is present in endosomes and activated by CpG DNA motifs and CpG-oligodeoxynucleotides (ODNs); TLR3 can be expressed at the cell surface or within endosomes, depending on the cell type, and is activated by dsRNA, which is formed during viral replication in host cells, and dsRNA analogs, such as polyI:C.

Upon endocytosis of a protein by APCs, the peptid-antigens are presented by MHC class II molecules to antigen-specific CD4+ T cells. When foreign (viral) proteins are present in the cytoplasm of a cell after transduction, the protein is digested into smaller antigenic peptides. The proteasome has a maintenance function here and controls degradation of different proteins present in the cytoplasm. Viral proteins are marked for degradation by covalent linking to multiple small polypeptides, named ubiquitins. After ubiquitination, the proteins unfold, the ubiquitin molecules are removed and the proteins are degraded by the proteasome. The peptides are then transported to the ER via transporter associated with antigen processing (TAP1 and 2) proteins, where they associate with the MHC class I molecules. The TAP proteins present in the ER regulate the ATP-dependent transport of the peptides from the cytosol to the lumen of the ER and are covalently linked to newly synthesized MHC class I molecules via the linker protein tapasin. Upon entry of a peptide into the ER by TAP, it binds to a specific groove in the class I molecules. This complex is then released from tapasin and free to leave the ER to be transported via the Golgi system to the cell surface. The α-chain/β2-microglobulin dimers, not linked to peptides, are very unstable and cannot be efficiently transported from the ER. In contrast, the peptid-bound MHC class I complex is structurally stable and will be transported to the cell membrane, where the peptides are presented and recognized by peptide-specific CD8+ CTLs. This cell-mediated immune response plays an important role in the elimination of viruses from the body.

IMMUNE RESPONSE AGAINST VIRUSES, VIRAL VECTORS AND TRANSGENES

The intensity and type of host immune response depends on the viral vector, the transduced cell or tissue type, the level of transgene expression, the immunogenicity and localization of the transgene and the immunologic competence of the host.

Viruses use host mechanisms to multiply. Cytopathic viruses multiply quickly and have to rapidly spread to other organisms before they cause cell death of the infected cell or before they are cleared by the immune system. Persistent viruses, such as herpes viruses, can exist latently for longer periods of time in infected cells and display minimal expression of viral genes, with the exception of genes absolutely indispensible for virus survival. These viruses can reactivate at any given moment and reinitiate production of virus particles[176,177]. However, as a general rule, the defense mechanisms of the host determine for both types of viruses the time period during which the virus can actively reproduce. Viruses with the ability to either prolong this period or evade immune recognition have increased probabilities of transmission and survival. Dealing with the anti-vector innate immune responses remains one of the major bottlenecks of gene therapy. Prudent strategies to limit the innate immune response include *ex vivo* gene transfer or *in vivo* delivery of a minimal number of viral particles. Strategies to limit the adaptive immune response against the vector and the transgene product use a two sided approach, in which simultaneously the immunogenicity of the vector cq transgene product is minimized by vector modifications (targeting and shielding) and/or restricted expression and the host immune system is suppressed with immune modulatory agents.

Adenoviruses

Adenoviruses (Ad) are icosahedral, non-enveloped double-stranded DNA viruses with 35-40 kb genomes[114,174]. There are currently 54 serotypes classified into seven groups (A-G) based on sequence homology and their ability to agglutinate red blood cells[178-180]. Adenoviruses can infect a wide variety of both dividing and quiescent cells, but only rarely integrate in the host genome[114]; they are easily purified to high titers of up to 10^{12} to 10^{13} vector particles per mL; they can accommodate 5-8 kb (first generation Ad vectors), 10-13 kb (second generation Ad vectors) to 36-37 kb (gutted vectors) non-viral transgenic material[178]; and the serotypes most commonly used for therapeutic purposes, i.e. species C Ad2 and Ad5 are well characterized. Most Ad virusus, species B excluded, make use of CAR as their primary docking receptor to infect cells[177]. Disadvantages of Ad include the presence of preexisting immunity in the majority of humans, low transduction of important gene therapy target cells due to low expression of CAR and innate toxicity upon intravascular administration as a result of complement activation, cytokine release and vascular damage, resulting in a systemic, possibly lethal, inflammatory response[179]. Cross-reacting antibodies against Ad are directed against the hexons present in the protein capsid and contain the generic antigenic component common to all mammalian Ad[176]. Serotype-specific sites on the hexons are responsible for induction of neutralizing antibodies. Adenoviral vectors based on different serotypes make use of their specific virus attachment receptors, which affects both vector tropism, and downstream signaling pathways, resulting in distinct host immune responses (Table IX).

Modification of the virus has resulted in different types of adenoviral-based vectors developed in recent years, resulting in the development of first generation (E1A and E1B deleted with or without partial deletions of the E3 genes), second generation with additional modifications in E2A, E2B, E3 and/or E4 regions, and helper-dependent (HD), high-capacity (HC) "gutted" or "gutless" vectors, stripped of all viral gene products[181,182]. To allow efficient packaging of the latter into the Ad capsid, a genome size between 27.7kb and 38 kb is required and can be obtained by including "stuffer" DNA sequences in the E3 region of the helper virus[183,184]. Another advantage of the "stuffer" DNA is that although it does not prevent homologous recombination, it does result in recombinant genomes unpackageable because of their large size, exceeding the packaging capacity of the Ad virion[32]. The oncolytic conditionally replicating adenoviral vectors (CRAd) were developed to target tumor cells, however, without targeting, oncolytic CRAd can only infect CAR-expressing tumors and efficacy of transducing other cancers is low[185]. A variety of molecular targeting and shielding strategies (see appendix B) have therefore been applied to develop CAR-independent oncolytic Ads, such as tumor-specific promoters (TSP) upstream of the E1A gene for specific expression in target cells or deletion of viral functions, dispensible in neoplastic cells, eg oncolytic Ad5-based vector ONYX-015 (*dl*1520) was created by the deletion of E1B-55K[186] and results in a vector that can replicate in p53-defective cells, but a 100-fold less in normal cells expressing functional p53[186]. Similarly, Oncorine H101

contains a deletion of E1B-55K and has been used as an oncolytic vector to treat nasopharyngeal cancers, glioma, pancreatic and ovarian cancers[4,187]. Gendicine, or Ad-p53, contains the p53 transgene in place of the viral E1 region under a Rous sarcoma virus promoter and has recently been approved for clinical use in China for the treatment of head- and neck squamous cell cancer in combination with radiotherapy[187,188], but has also been tested in a wide range of other solid tumors, such as lung cancer, liver cancer and cancerous ascites[188].

Immune response against adenovirus and adenoviral vectors

Adenoviruses have been shown to precipitate immune responses through TLR-dependent (pDC) and TLR-independent mechanisms (Kuppfer cells)[189]. These immune responses against Ad are rapid and prevent efficient re-administration of the vector. Induction of cytokines, such as IL-6 and TNF-α, appearing in response to Ad infection, is mediated through TLR9, an endosomally located TLR shown to recognize CpG DNA. Human cell lines expressing TLR9, although permissive to both CAR- and CD46-interacting Ad serotypes, display preferential activation of TLR9 by CD46-interacting serotypes[190], presumably due to their tendency to reside longer in the late endosomal compartments than CAR-interacting serotypes[191]. The route of Ad entry may substantially contribute to the type of innate immune response initiated by the TLR pathways. In addition, genomic Ad DNA was shown to be able to induce of IL-6 and TNF-α[181]. The cytokines and chemokines most important for the anti-adenoviral immune response are IFNα and β, IL-1, IL-6, IL-8, IL-12 and TNFα in humans and KC and MIP-2 in mice. Ad virions initiate rapid host transcriptome responses independent of Ad transcription, DNA replication and/or protein expression. Although empty capsids appear capable of inducing immune responses, this may not be the case for all cell types. Induction of Type I IFNs by the Chimpanzee AdC68 is independent of viral transcription and replication and substantially inhibits vector-derived transgene expression[97]. Innate immune signaling against Ads is dependent on endosomal trafficking and/or rupture[184,185].

In spite of the E1 deletion, the first generation vectors are "leaky" and viral genes are expressed at a low level in transduced cells, resulting in direct toxicity and immunogenicity of the viral gene products[192]. Deletion of E3 is not always advantageous, as expression of E3 genes can diminish anti-vector immune responses and increase persistence of transgene expression[191]. In situations, where short-term gene expression is sufficient to achieve a therapeutic effect, first generation vectors are attractive choices. Activation of cytotoxic T cells (CTLs) in response to newly synthesized viral antigens and transgene product leads to destruction of vector-transduced cells and loss of transgene expression[182]. The major effectors in elimination of transduced cells are the MHC class I restricted CD8+ CTLs, whereas the CD4+ T cells by secretion of IFN-γ sensitizes virus-infected cells to CTLs through upregulation of MHC class I expression and allow the development of a fully competent CTL response and contribute to the formation of neutralizing antibodies[193,194]. Second generation Ad vectors were developed to decrease activation of cellular and humoral immune responses[192]. The fact that high-capacity helper-dependent Ad vectors, devoid of any viral genes, induce remarkably similar immune responses as conventional Ad, including a prominent IFN type I response within 6 hours after administration and upregulation of IFN-responsive genes, suggests that capsid structures, rather than leaky viral gene expression, are sufficient to induce an innate immune response[195]. However, the period of time during which the immune system can recognize cells infected with the HDAd vector is very limited and only takes place when antigens derived from HDAd capsid proteins are transiently presented on MHC class I molecules during the disassembly of the injected vector. Once the input virion proteins have been metabolized, the HDAd transduced cells become invisible to anti-Ad specific T-cells[196].

Human adenoviral viral vector serotype 5 (Ad5) is presently the primary viral vector used in gene therapy trials. However, binding of the Ad5 fiber to CAR results in the activation of ERK1/2 and JNK MAPK kinases and subsequent upregulation of transcription of pro-inflammatory genes, such as IL-8, GRO-α, GRO-γ, RANTES and IFNγ-inducible protein (IP-10). Activation of complement by Ad5 results in opsonization by macrophages and Kuppfer cells and in the release of inflammatory cytokine and chemokine production and is required for the induction of neutralizing antibodies[197,198]. Neutralizing antibodes against anti-Ad reaches its peak 7-14 days post-virus treatment, whereas the anti-transgene responses peaks somewhat later around days 23-28[197].

Many pathways work together during the inititiation of the acute anti-Ad immune response and require attentiona: inhibiting one of these, will diminish the immune response, but is not likely to be sufficient to provide complete protection against Ad-immunity. Binding of the Ad-vectors to complement, PRRs, erythrocytes[199],

platelets[200] and blood clotting factors, eg factor X[201] and scavenging of Ad by macrophages and Kuppfer cells[202], have been implicated in facilitation of the innate immune response.

Adeno-associated virus

The adeno-associated viruses (AAV) are non-pathogenic, single-stranded DNA viruses and naturally replication-defective, ie they depend on co-infection of an unrelated helper virus for productive infection. Several viruses such as adenovirus, herpes virus, but also vaccinia and human papilloma viruses can provide the helper activities required for AAV growth[203]. Currently, twelve human AAV serotypes are known and more than a hundred have been isolated from non-human primates. AAV-based vectors are becoming increasingly popular due to its non-pathogenicity and relative low immunogenicity, as well as its heat stability and resistance to solvents and to changes in pH and temperature[204]. Recombinant AAV vectors consist of merely 300 nucleotides from the original viral sequence in the form of non-transcribed ITRs, which is a major step forward in ensuring its safety for human clinical applications by reducing the risk of recombination with wild-type virus. The rAAV vectors can be used to target a wide range of different host and cell types due to their broad tropism and their capacity to transduce both dividing and non-dividing cells, while their lack of viral coding sequences reduces the risks of eliciting an important cellular immune response and thus prolongs expression of transgenes *in vivo*. The latter is a major requirement for gene therapy of some inherited genetic diseases.

The most commonly used rAAV vector for gene therapy is based on AAV-2. In the absence of helper virus, AAV serotype 2 can become latent and integrate site-specifically into chromosome 19q13.4[205]. This specificity of integration is determined by the presence of the ITRs and the rep gene. However, recombinant vectors lose this specificity due to deletion of the rep gene and integrate randomly[116]. AAV-2 gains entry into target cells through binding to heparan sulfate proteoglycan (HSPG)[193] and one or more co-receptors including $\alpha_v\beta1$ and $\alpha_v\beta5$ integrins, FGF-R1, HGF-R and the laminin receptor (see Table X).

AAV-based vectors

In rAAVs used for gene therapy, the ORFs are removed and replaced by the transgene and regulatory elements flanked by the ITRs. The vectors are propagated in cell lines that provide the AAV genes *in trans* and provide helper virus functions through the E1, E2, E4 and virus-associated RNA of adenovirus[203]. Drawbacks of the single stranded (ss) AAV vectors include a restricted packaging size (4.7 kb)[194], limiting the applications of rAAV to relatively small gene diseases, and in case of rAAV-2 based vectors, inefficient transduction of certain clinically important tissues, such as liver and muscle, and the existence of anti-AAV-2 neutralizing antibodies[206]. In addition, inefficient intracellular viral processing[207] and second-strand synthesis[208], which have been identified as rate-limiting factors in AAV gene expression, could be optimized. Several novel techniques, such as *trans*-splicing AAV vectors, are being developed to increase the genome capacity for AAV and enhance gene expression[209]. This approach entails the co-administration of two independent *trans*-splicing rAAV vectors encoding complementary segments of a large therapeutic transgene cassette and intron donor and acceptor signals and allows delivery of therapeutic genes up to 9 kb in size. However, the *trans*-splicing vectors are less efficient than the rAAV vectors. An alternative approach is the development of self-complimentary AAV vectors, which circumvent the delay in transgene production expression associated with the conversion from ss to ds DNA, and shows superior transduction of certain cell types, but have a packaging capacity limited to half of that of the traditional ss AAV vectors.

Immune responses against AAV and AAV-vectors

The host response against wild-type AAVs is likely partially determined by the helper virus, which induces the innate immune response and facilitates the immune response against AAV, but is also strongly dependend on the level activation of the immune system. Other parameters include pre-existing immunity, the route of administration, the kinetics of expression, the dose, the serotype, the host species, in case of vectors the immunogenicity of the transgene and importantly, the ability to transduce or infect antigen-presenting cells[210]. Since AAV vectors themselves appear to lack PAMPs, they cannot activate TLRs[211]. Although AAV2, in contrast to AAVs from other clades, can infect DCs directly through binding of HSPG[212], an unidentified post-entry block inhibits successful transgene product expression and upregulation of co-stimulatory molecules and MHC classes I and II[213,214]. As a result, AAVs are not able to induce maturation of human DCs and production of type I IFNs[215]. In contrast, in mice, type I IFN responses can be induced by AAV through activation of mouse plasmacytoid DCs (pDCs) via TLR9-MyD88 and are crucial for the activa-

tion of the CD8+ T-cell response[216]. However, AAVs can induce capsid and transgene product-specific immune responses, including neutralizing antibodies[217,218]. One reason that AAV2 vectors induce an immune responses is that they uncoat relatively slowly allowing for a prolonged period of time during which processed AAV2 capsid peptides are presented in the context of MHC class I to antiviral T-cells[219-221]. Pseudotyping the particles with capsids from AAV6 or AAV8, which in contrast to AAV2 do not persist as encapsidated molecules[219], or the use of AAV5 vectors, which uncoat more rapidly than AAV2 capsids[196], results in better transduction and decreased inflammation. Short-term immune modulation during the uncoating of AAV2 could therefore be helpful in preventing presentation of capsid epitopes by MHC class I molecules and the duration of the immune suppression would thus be directly linked to the half-life of the viral capsid proteins[196]. Other mechanisms involved in the clearance and induction of immune responses against AAV include inhibition of AAV replication by APOBEC3A (hA3A)[222], cross-presentation of antigens[211] and macrophage activation by complement[223]. AAV capsid binding to C3 complement proteins enhances AAV uptake into macrophages and macrophage activation and, indeed, intravenous treatment of DBA/2 mice with AAVlacZ resulted in a rapid, but short-lived induction of TNF-α, RANTES, IP-10, MIP-1β, MCP-1, and MIP-2 mRNAs in the liver, which was dependent on the presence of Kuppfer cells, and a transient infiltration of neutrophils and CD11b+ cells[224]. Although binding of AAV to the co-factor complement regulatory protein factor H, results in conversion of C3b in iC3b and abrogation of further complement activation[223,225], the AAV capsid-iC3b complexes are still subject to some immune vigilance and binding of the complement receptor complex with the B-cell receptors (BCRs) activates B-cells. The expression of viral peptides by B-cells in the context of MHC class II molecules results in stimulation of CD4+ Th cells. Interactions between CD40L on the Th cells and CD40 on B-cells then leads to clonal expansion and production of antibody-producing plasma cells and memory B-cells. Not only impairs a deficiency in C3 protein or complement receptors CR1/2 the humoral response to AAV significantly[223,225], also the role of co-stimulation and Th cells is important for an optimal immune response and CD8 cellular immunity to AAV vector capsids is ablated in the absence of CD4, CD40L, or CD28[217,226]. Approximately 30-60% of the humans carries neutralizing antibodies against AAV2 and cross-reacting antibodies against many other serotypes[227,228]. The extent of cross-reactivity between some AAV serotypes appears to be species specific and dependent on tissue type and route of administration. In mice, even low titers of neutralizing antibodies were able to impair the efficacy of AAV-mediated gene transfer to liver[229] or brain[81] and in clinical trials, the induced IgG response to the AAV capsid was shown to be inversely proportional to the level of pre-existing anti-AAV antibody and independent of the vector dose[230]. However, in immune privileged sites, such as the brain, it was shown that circulating anti-AAV2 antibodies can inhibit AAV2-mediated, but not AAV5-mediated gene transfer[81]. In contrast to the long-term expression of therapeutic levels of fIX after treatment with AAV vector in mice and dogs[231-233], despite the formation of anti-hF.IX antibodies[234], AAV2-fIX in men with severe hemophilia B at a dose of $2x10^{12}$ vg/kg resulted in only short-lived therapeutic levels of fIX expression[49] likely as a result of an anti-AAV capsid CD8+ T-cell response[235]. It was shown in hemophilia B dogs that not only the total vector dose administered plays an important role in the formation of antibodies[236], but also the vector dose per injection site[234]. Analysis from immunoglobulin classes showed that noninhibitory antibodies consisted of IgG2 only, whereas inhibitory antibodies were found to be both IgG1 and IgG2. In addition, it was shown that AAV1 vectors were much more potent in induction of inhibitory antibody formation than AAV2-CMV-F.IX[234].

Also AAV vectors used as vaccine carriers were shown to induce transgene-specific T- and B-cell responses, although mild in comparison to Ad vaccines, and may be due to lack of transgene product expression by DCs[213]. Normal human subjects were shown to carry wt AAV-specific memory CD8+ T cells, which can expand upon re-exposure to capsid antigens and induce a rapid clearance of transduced cells[56,218,235]. Pseudotyping of AAV2 with capsid proteins from other less prevalent or animal type serotypes can circumvent pre-existing immunity. In a clinical trial for AAT deficiency, both neutralizing antibodies and cross-reacting antibodies against other serotypes increased after intramuscular administration of AAV2/1-AAT[237]. Despite induction of neutralizing antibodies, long-term low level expression of AAT was found in all patients, suggesting that after intramuscular injection some transduced may escape immune-mediated elimination. In contrast, animal models, such as the *mdx* mouse model, display substantial immune responses upon intramuscular injection of AAV, possibly due to leakage of neoantigens from affected muscle cells[238]. Administration of AAV vectors to immune-privileged sites, such as the brain, in adults appears not to be negatively

affected by (pre-existing) immunity. In a phase I trial to test the safety of a single local infusion of up to $0.5x10^{11}$ vg AAV2-GAD (AAV-glutamic acid decarboxylase) in patients with Parkinson's disease (PD), an increase in anti-AAV2-antibodies or anti-GAD antibodies could not be detected[239]. In contrast, in another phase I trial for PD, 4 out of 6 adult patients injected with $5.4x10^{11}$ vg of AAV-NTN (Neurturin, CERE-120) at multiple sites in the putamen, showed an increase in anti-AAV2 antibodies, but no humoral response against Neurturin, whereas 6/6 patients, who received a lower dose of $1.3x10^{11}$ vg did not show any meaningful increases in anti-AAV or anti-Neurturin antibodies[240]. A study in young children with Canavan disease, showed the appearance of detectable neutralizing antibodies to AAV in 3 out of 10 children after treatment[241]. The reason for the discrepancy between these studies is not clear, but may be related with the age of the patients, the total viral dose of the vector, whether the patients received a single or multiple injections and whether the patients suffered from postoperative complications, such as fever and/or hematomas.

Thus, although mild, immune responses against AAV can hamper the efficacy of the treatment and methods to circumvent some of the major key players in the immune response include the use of alternative serotypes[145], pseudopackaging[149] and the selection of immune-escape mutants[242]. Although these methods can all delay or diminish the primary immune response, they cannot completely prevent induction of an immune response and therefore, other methods such as induction of tolerance, for example by increasing Tregs or targeting liver cells, using a different ROA and immune modulation may be needed for optimal use *in vivo*. For AAV, immune modulation focused on Kuppfer cell depletion, complement inactivation or blocking of the CD8+ T-cell response could considerably improve transgene expression. Interestingly, it was shown that the anthracycline antibiotics, such as doxorubicin, daunorubicin and epirubicin, and platinum compounds, which are both commonly used as cancer chemotherapeutics and have an immune suppressive effect, can augment AAV2 mediated gene transfer, by promoting nuclear accumulation of AAV2[243,244].

Immune response to other parvoviruses

Some rodent parvoviruses (PV), currently explored for their use as an oncolytic virus, are rat H-1PV, an autonomously replicating, non-integrating virus[245] and parvovirus Minute Virus of Mice (MVMp)[246]. After translocation to the nucleus, H-1PV is dependent on proliferation and transformation of host cells for conversion of ssDNA into dsDNA, gene expression and cytotoxic activity (J. Rommelaere, ESGCT 2009). The PV adaptor protein NS1 affects cellular kinases and is responsible for oncolysis. Although H-1PV is infectious for normal mammal cells, it does not result in cell killing or significant pathology in humans. Recognition of H-1PV and MVMp by PRRs results in a type I IFN response and clearance of the virus. The inability of transformed cells to induce such an innate immune response, allows completion of the lytic cycle and results in enhanced oncotropism. Oncolytic virotherapy was shown to be feasible *in vitro* and *in vivo* immuno-deficient animal cancer models for glioblastoma multiforme[247], pancreatic cancer[248] and lymphomas[245]. The anti-tumor effects of H1-PV can be improved by arming the virus with immunogenic CpG sequences or immune-stimulating molecules, such as MCP-3, or by combination treatment with the antibiotic norfloxacin[249]. H-1PV is currently tested in a phase I/Iia study for patients with glioblastoma multiforme.

Herpes Viruses

Herpes viruses are characterized by their tendency to remain latent after primary infection and reactivate at later time points. Herpes Simplex Virus (HSV) is an enveloped dsDNA virus with a genome size of 152 kb, encoding 84 viral genes, approximately half of which are dispensable for replication *in vitro*, but may affect virus virulence *in vivo*.

Replication-competent (RC) vectors, although attenuated, carry the risk for pathogenesis, mutation or recombination, shedding and germ-line transmission[154]. The replicative ability of ICP34.5-deleted HSV is cell type and state dependent[250]. In general, dividing cells support replication of ICP34.5-null HSV, whereas non-dividing cells cannot support its growth. In non-permissive cells, failure to express ICP34.5 results in a defect in virus maturation and transportation from the nuclei of infected cells[251]. In contrast, malignant cells allow selective replication of ICP34.5-null HSV-mutants[252].

The first generation oncolytic HSV vectors contain a single gene mutation to prevent replication in non-dividing cells. The HSV-1 F based, highly neuroattenuated strain R3616 contains a deletion of both copies of ICP34.5 and has a good safety profile as demonstrated by the lack of encephalitis in an experimental glioma mouse model, while the virus can still replicate in actively dividing cells and exert antitumor effects[253]. Strain HSV1716, derived from HSV-1 Glasgow strain 17+, has a similar ICP34.5-deletion[254] and was tested in a Phase I clinical trial demonstrating lack of toxicity in immune compe-

tent patients[255,256]. However, intraventricular treatment of nude mice with even a low dose of 10^3 pfu HSV1716 demonstrated significant lethality[257] and indicate that treatment of patients who are immune compromised may suffer from severe viral pathogenesis.

Second generation HSV vectors contain multiple mutations, to prevent reversion of the strains into wt and ensure a better safety profile. Vector G207, was derived from the R3616 by insertion of the lacZ gene into the UL39 gene, encoding the large subunit of RR (ICP6)[258]. In a Phase I and Phase Ib study, it was demonstrated that multiple doses of G207 were well tolerated, with no signs of toxicity or encephalitis[259,260]. Animal studies demonstrated a localized distribution of G207 viral DNA after injection in the brain and absence of viral DNA in excreta up to one month after injection[261].

The replication-defective (RD) HSVs lack genes essential for *in vitro* viral replication, such as the immediate-early (IE) genes encoding ICP4 and ICP27 shortly after viral entry[262]. The first generation defective genomic vectors contained deletions in a single encoding ICP4[263], but were still neurotoxic in culture. Second generation RD vectors, deleted for various combinations of IE genes, showed reduced cytotoxicity in comparison to the first generation and allowed long-term gene expression in neurons[264].

Amplicon vectors are similar to HSV-1 particles in structure, immunogenicity and host-range, but do not carry any viral genes, nor induce synthesis of viral genes, rendering it fully nontoxic and nonpathogenic[265] and can accommodate foreign genes up to 130 kb of a total packaging capacity of 152 kb[265,266]. Amplicon plasmids are dependent upon helper virus function to provide the replication machinery and structural proteins necessary for packaging amplicon vector DNA into viral particles[267]. Although the risks of reactivation, complementation or recombination with latent HSV-1 are considerably lower than with any of the other HSV-based vectors, its high-titer production is far more difficult, with titers ranging in the order of 10^7 to 10^8 virus particles/mL[267].

Immunogenicity of HSV and HSV-based vectors

Wild-type HSV-1 and HSV-2 are highly immunogenic and induce rapid activation of the innate immune system as well as cellular and humoral immunity against HSV antigens and transgenes. More than half of the population already possesses neutralizing antibody activity against viral envelope glycoproteins gB, gD and gH-gL[58,268]. CD4+ T-cell responses are broad and directed against immediate early, early and late proteins, envelope proteins gB-E and gH, tegument, capsid and nonstructural antigens present within infected cells[58]. Epitopes recognized by CD8+ cells include gB, RR1[269], ICP0, tegument proteins VP13/14 and VP22[270]. However, some subjects may display a persistent anti-HSV T-cell response, in the absence of detectable antibodies, indicating either undetected infection or acquired immunity in the absence of infection[271]. The main difference between immune and non-immune individuals appears to be the velocity and the magnitude of the acquired response. As a first line of defense, factors in saliva[272], tears and blood[268] bind and attempt to neutralize HSV. Rapid activation of the complement cascade[273] is followed by local inflammation, infiltration of neutrophils, macrophages, T-cells and NK-cells[274]. The type I IFN response is mediated by activation of TLR2[275], TLR3 and TLR9[276]. Viral particles, which succeed in entering the neuronal axons, travel by means of retrograde transfer to sensory ganglia, where they remain out-of-reach for the host adaptive immune responses. Cytokines affecting the course of the HSV infection include IFNα, β, γ, IL-1, -2, -4, -5, -6, -10, -12 and -23 and TNFα[277]. Initially, IFNα/β and TNFα are produced and effectively limit the early acute replication and spread of HSV[278] and activate macrophages. Shortly thereafter, IL-12 in consert with other of the above cytokines induces production of IFN-γ in CD4+ Th1 cells, CTLs and NK cells[279,280]. IFNγ can be detected throughout the infection and is associated with improved survival, resistance from encephalitis and prevention from reactivation[274]. Inactivation of the IFNs leads to elevation of viral replication and an increased viral load[281]. IL-23, produced by dendritic cells, is also upregulated during infection[282], stimulates IFNγ and plays a role in the control of memory T-cells[283] residing in regional lymphnodes[284], as well as in maintenance and induction of the type I cytokine and Th1 response[285]. Other mediators of the Th1 response include IL-1β, which regulates inflammatory responses, IL-6, which has predominantly antiviral effects and TNFα, the expression of which continues during latency[274]. Exposure of macrophages to IFNγ results in induction of the enzyme inducible nitric oxide synthetase (iNOS) and production of NO, which has a strong antiviral activity, but when overproduced might result in damage to DNA, proteins and lipids in host cells and tissues[279]. The Th2 cells are the primary producers of IL-4 and IL-13, which repress the induction of IL-12 and can halt the positive feed-back loop of IFN-γ production to prevent an ongoing pro-inflammatory response and potentially harmful actions of macrophages and NK cells[279]. Whereas CD4+ T-cells and NK-like cytotoxic responses are present

during all stages of infection, the infiltration of CTL is associated with actual viral clearance[286].

The same host responses that are activated by wild-type HSV-1 infection are also operational in limiting the anti-cancer effects of HSV-1-based oncolytic vectors[268]. Within hours of exposure, activation of the innate immune reponse results in upregulation of anti-viral cytokines and chemokines. It was demonstrated that plasma factors from naïve athymic and immunocompetent rats and humans can *in vitro* impede transduction with the oncolytic replication-conditional HSV mutant hrR3 and that this activity could be quenched by mild heating of the plasma[67]. Treatment of rats with cobra venom factor (CVF) to deplete complement factors or with cyclophosphamide, which decreases neutrophil levels in the peripheral blood and inhibits immunoglobulin production by B-cells, resulted in increased transduction efficiency of brain tumors after intra-arterial injection of hrR3[68,268]. Mice with intracranial melanoma were treated with intratumoral injections of HSV1716 (see above) and showed an anti-tumor and anti-vector CTL response and a tumor-specific proliferative T-cell response, but absence of a (neutralizing) antibody response against the tumor or the vector and it was suggested that an optimal antitumor effect required the presence of an integrated, complete immune response[287]. Despite pre-existing antibodies against HSV, melanoma patients treated with intralesional injection of HSV1716, showed viral replication within the boundaries of the tumor and tumor flattening, without shedding/reactivation of endogenous latent virus or effects on serum IgG or IgM levels[288]. Although in both HSV seronegative and seropositive patients intratumoral injection of HSV1716 showed viral replication in high grade gliomas without causing toxicity, injection resulted in increased serum levels of IgG and IgM and seroconversion two seronegative patients[289]. As a safety measure, however, some level of neutralizing activity against HSV-1 may be partially protective in inhibiting spread of a replicating virus.

RD-HSV vectors own a virtually intact HSV genome and immune responses may arise from viral particle components, co-purified packaging debris, low-level *de novo* viral gene product expression and expression of the transgene[267]. As is the case with the OV, preexisting immunity does not appear to negatively affect immunity elicited against RD-HSV-1 vaccine vectors or transgenes[290]. However, no detailed assessments of any of these confounding factors have been performed up to date. More information is present on immune responses against amplicons. The earlier amplicons, contaminated with helper virus allowed low level expression of viral proteins[267]. These amplicons induced a strong inflammatory response with MHC class I and II expression, T-cell activation and an influx of macrophages[291]. Although mice injected stereotacticly with helper-free preparations of β-galactosidase-expressing amplicon (HSVlac) displayed a similar innate immune response to mice injected with preparations contaminated with helper virus, this response fully resolved within 5 days, demonstrating that helper virus-free amplicon preparations exhibit a safer innate immune response profile[292]. Furthermore, it was shown that infection with HSV amplicons triggers an IRF3 and IRF7-dependent, but TLR-independent antiviral response, which results in only a mild and contained type I IFN response[293].

As mentioned before, the pathogenic effects of replication competent HSV vectors, when administered to athymic nude mice are major and can result in lethality, depending on the dose of the vector. In addition, immune compromised patients, in particular patients with defects in cell-mediated immunity may suffer extensively from a HSV infection, resulting in severe local infections, encephalitis, and even generalized infection. Thus, immune modulation in a patient treated with a RC-HSV vector should be carefully chosen and preferably not target cellular immunity, but for example the IFNα and β response, which limits the early acute replication of HSV. Rather than suppression of the innate or cellular immune response, stimulation of elements regulating the Th2 reponse can be an alternative route to achieve immune modulation. Broberg *et al.* used linomide to facilitate viral infection and found an increased expression of IL-4 and IL-10 transgenes, but no effect on the clinical course of infections[294].

Vaccinia virus

The poxviruses are the largest known DNA viruses and are distinguished from other viruses by their ability to replicate entirely in the cytoplasm of infected cells: Since poxviruses do not require nuclear factors for replication, they can even replicate with little hindrance in enucleated cells. Vaccinia virus (VACV) belongs to the orthopoxvirus subfamily and has been used as a vaccine for the eradication of smallpox[295]. Important strains of VACV are derived from the New York City Board of Health (NYCBH) strain, which was used to develop the Dryvax and ACAM2000 vaccine strains, as well as the Western Reserve (WR) strain[79]. NYVAC is another attenuated form of VACV derived from the Copenhagen vaccine strain and has a deletion of 18 open reading frames from

the viral genome[296,297]. VACV is highly immunogenic and induces a strong CTL and long-lasting neutralizing antibody response. This long-lasting response was the basis for the use of VACV as a vaccine for the eradication of smallpox and has resulted in extended experience with Vaccinia and a thorough knowledge of its side effects. VACV has also been used as a vaccine vector to present antigens from other pathogens and in the treatment of cancer[298-301] and new strategies include methods to boost rather than decrease the immune response, eg by using different poxviruses[302], or through co-expression of combinations of co-stimulatory factors[303]. The rationale behind the use of Vaccinia for cancer treatment is clear: VACV replicates and lyses cells rapidly; has a broad tumor tropism and does not require specific receptors for cell entrance; does not integrate into host DNA; displays efficient spreading, allowing systemic administration; can be treated, if needed, with experimental anti-viral agents[304] or Vaccinia immunoglobulin[305]; and can accommodate large genes up to 25 kb[295]. Vaccinia vaccine strains inherently target tumors[306], as these are more susceptible to viral replication due to blocks in apoptosis and deregulation of the cell cycle and conceal the virus from the immune system[295]. In addition, Vaccinia expresses an EGF homologue, Vaccinia growth factor (VGF), that activates the EGFR-Ras pathway and anti-viral agents designed to block this pathway[307] inhibit Vaccinia replication. Oncolytic Vaccinia vectors with increased tumor-specificity can be constructed by deletion of genes expendable for viral replication in tumor cells, such as the TK[308,309] and VGF. The TK- and VGF-deleted vvDD vectors display selective replication in tumors with activated EGFR-Ras pathways[310]. Increased tumor-selective replication can be achieved by deleting several other genes, including two serpins and an inhibitor of cytochrome c release[295].

Immunogenicity of Vaccinia

Vaccinia-induced cell lysis results in the release of PAMPs and both virus- and tumor-associated antigens and can be used to boost the immune responses against a tumor[302,303]. In non-immunized patients, circulating virus is encountered by complement and reticulo-endothelial cell-based mechanisms, leading to phagocytosis of viral particles by tissue macrophages or liver Kuppfer cells, induction of type I IFNs through TLR-dependent and independent mechanisms[311], activation of NK cells by type I IFNs[312] and clonal expansion of CD8+ T-cells through direct signaling of TLR2-MyD88[313]. For recovery and protection from secondary infection, B-cell function and production of neutralizing antibodies are critical[314], whereas type I IFNs[315] and CD8 T-cell effector functions are not essential[166]. The rapid and robust immune response in previously immunized patients can considerably decrease the anti-tumor effects of Vaccinia and must be circumvented. Although systemic immune suppression or B-cell depletion can enhance viral infection of tumor cells, it may simultaneously decrease the immune response against the tumor. Rather, simultaneous shielding of the vector from the immune system, while maintaining the host immune responses against the tumor, can enhance the efficacy of oncolytic Vaccinia virus[40]. A single systemic dose of replicating, double deleted vaccinia virus vvDD-EGFP inhibited growth of malignant glioma cells in athymic mice and increased survival in immune competent mice. Combination treatment of vvDD with rapamycine and cyclophosphamide enhanced viral replication and further increased survival[40]. Cyclooxygenase-2 (Cox-2) inhibitors represent a new class of non-steroidal anti-inflammatory drugs that reduce inflammation and can attenuate antibody production by inhibiting antibody induction[316]. Furthermore, it was shown that treatment with Cox-2 inhibitors allowed the repeated administration of Vaccinia virus for the treatment of ovarian cancer[317]. Simultaneously, other groups attempt to use the strong cytotoxic T-cell response induced by Vaccinia to induce a local anti-tumor response[295].

Retroviruses

Retroviruses are divided into two subfamilies, the *orthoretrovirinae*, including gammaretroviruses (MLV, HTLV-1 and HTLV-II) and lentiviruses (HIV-1, HIV-2 and SIV-2), and the *spumaretrovirinae* (foamy viruses, FV)[20].

Gammaretroviruses

In the early eighties, the development of the 'traditional' retroviral vectors from MLV commenced. One of the major drawbacks of the onco-retroviral derived vectors is their inability to transduce non-dividing or quiescent cells and their preferred integration in promoter regions, which may lead to insertional mutagenesis, as was shown in a recent clinical trial for X-linked SCID[318]. In the cases of SCID-X1 it is now believed that an initial aberrant expression of an oncogene (mainly LMO2) led to proliferation of specific clones and the addition of other genetic events, eventually resulting in leukemic transformation[319].

Immune responses to lentiviral vectors

In contrast to the retroviral vectors, the lentiviral vectors (LV) have been shown to efficiently transduce dividing

and non-dividing cells and therefore have a better safety profile than the retroviral vectors. The most commonly used LVs are derived from proviral DNA of the human immune deficiency virus type I (HIV-1). The LV vectors, similar to high-capacity Ad vectors and AAV vectors, do not encode viral genes. In contrast to the HCAd and AAV vectors, which despite a lack of expression of viral genes induce an effective anti-vector immune response due to highly immunogenic epitopes on the viral capsid, immune responses against LV vectors appear to occur only in the presence of an antigenic (foreign) transgene[69]. This difference between the induced immune response against HCAd, AAV and LV vectors, was suggested to be mediated by differences in viral capsid turnover: slow uncoating of antigenic capsid proteins, such as observed with AAV, allows longer interaction with the immune system to direct an anti-capsid response, whereas rapid capsid turnover, such as with LV, prevents recognition of immunogenic epitopes by activated effector T-cells, rendering the transduced cells invisible to the immune system[69]. LV vectors are a promising tool for gene therapy of CNS diseases due to the ability to transduce quiescent cells. Injections of LV-GFP in the CNS of Sprague Dawley rats or systemically did not induce an inflammatory response, but rats immunized subcutaneously with LV-GFP or LV-fIX displayed a significant immune response against lentivirus virion (p17 and p24) and envelope (VSV-G) proteins[69]. Although LV-mediated gene transfer allowed sustained transgene expression, even in presence of a pre-existing immune response against the vector, peripheral immunization against the transgene can lead to decreased transgene expression and increased inflammation with an increase of CD8+ T-cells in the CNS. Nevertheless, despite relatively low level immunogenicity of the parental virus, persistent high-level LV-mediated transgene expression has been difficult to achieve due to the fact that LV particles are pseudotyped with commonly used envelopes, such as VSV-G, which transduce and activate APCs (DCs) more efficiently than wild type HIV-1. *In vivo* transduced DCs display a mature phenotype, produce TNF-α and stimulate activation of the adaptive humoral and CTL response to the transgene[320,321]. The lentiviral activation of DCs was found to be mediated by TLR3 and TLR7[320]. Importantly, although the potential immune response against LV is of no great concern, stable transduction of cells and long-term expression of transgenes may elicit a potent anti-transgene immune response, which may result in clearance of transduced cells, particularly if the transgene is foreign to the host. Although unwelcome when long-term expression of transgenes is needed, the ability of LV vectors to efficiently transduce DCs and induce an anti-transgene immune response can be efficiently used as a tool to stimulate antigen-specific CTLs for cancer immunotherapy[322-325], or as a vaccine vector[326].

Integration deficient lentiviral vectors (IDLV) carry mutations in integrase, preventing proviral integration into the host genome and resulting in episomal persistence of vectors in transduced cells[327]. Due to the lack of replication signals, the lentiviral episomes are gradually lost in dividing cells resulting in transient gene expression, but remain stable in quiescent cells. From a safety perspective IDLVs are preferred, as they display a much reduced risk for insertional mutagenesis as well as for the generation of replication competent recombinants.

Foamy Viruses

Although foamy viruses share their basic genetic order of LTR-gag-pol-env-accessory genes-LTR with the gammaretroviruses and lentiviruses, they differ with respect to their replication mechanisms. Most notably is the feature to reverse-transcribe the RNA pre-genome late in replication before the virus buds from the cell membrane[328]. Like the the gammaretroviruses, they are able to integrate into the genome, but the FV vectors display a distinct integration profile[329,330]. The FV vectors do not integrate preferentially within genes, despite a modest preference for integration near transcription start sites and a significant preference for CpG islands[329]. FV vector integrations in human hematopoietic cells were shown to occur both in introns and intergenic regions[331]. Humans are not natural hosts of FV and do not have any pre-existing immunity[332].

Other viruses

Reovirus

Reovirus is a naturally occurring, non-pathogenic, double-stranded RNA virus. Attachment of reovirus to target cells is mediated by the reovirus sigma1 protein. Junctional adhesion molecule-1 (JAM-1) is a serotype-independent receptor for reovirus, and sialic acid is a coreceptor for serotype 3 strains[333]. After binding to receptors on the cell surface, reovirus is internalized via receptor-mediated endocytosis. Viral infections of respiratory and gastrointestinal tract the may occur in childhood[334] without causing clinical disease[335], resulting in a high prevalence of neutralizing anti-reovirus antibodies in adults.

In non-transformed cells, infection with reovirus results in phosphorylation of cellular PKR (dsRNA-activated protein kinase) and arrest of viral protein translation and replication. In contrast, in Ras-activated cells, PKR remains unphosphorylated and is incapable of aborting viral translation, replication and cytolysis[336]. Therefore, Reoviruses are able to selectively kill cells with an activated Ras signaling pathway, which can occur through Ras mutation or aberrant expression of upstream mitogenic signals, such as overexpressed or mutated receptor tyrosine kinases[337].

Since RT3D is in essence a non-modified wild-type virus (ie it carries no transgenes), immune responses are only directed at the virus itself. Immune responses are mediated by RIG-1 and MDA-5, although either helicase is dispensible for innate immune signaling[338]. In animals a neutralizing antibody response occurs within 3-7 days and plateaus at day 9[14]. In a clinical trial, it was shown that even heavily pretreated patients with advanced cancer were capable of inducing a dynamic immune response during treatment with Reovirus Type 3 Dearing (RT3D)[335]. Before Reovirus treatment, CD3+CD4+ levels in patients were relatively decreased, whereas CD3+CD8+ levels were relatively increased in comparison to normal subjects. Levels of CD56+ NK cells in addition to both types of lymphocytes rapidly increased in response to reoviral therapy[335]. Inflammatory cytokine responses differed between patients and combined Th1 and Th2 cytokine responses were observed in 8 (38%) patients, as well as a cyclical increase in IL-5 (4 patients), IL-2 (3 patients) and IL-6, IL-8 and IL-12p40 in 2 patients[335]. Patients developed high titers of anti-Reovirus neutralizing antibodies despite previous chemotherapy and/or radiotherapy and reached plateau levels after 7-14 days[335,339]. Promising data from animal studies showed the efficacy of Cyclophosphamide in blunting the neutralizing antibody response against RT3D, but demonstrated that high doses of Cyclophosphamide may result in unwanted replication of virus in normal tissue and toxicity[14]. In addition, although non-pathogenic in healthy persons, in immune compromised animal models, such as immune deficient and neonatal mice, RT3D may cause severe neurovirulence and death[340]. RT3D is currently undergoing extensive evaluation in phase I and II clinical trials, either as a single agent or in combination with radiotherapy or cytotoxic chemotherapy.

Measles virus

Measles virus (*Morbillivirus*) belongs to the family of *paramyxovidae*, as do respiratory syncytial virus (*Pneumovirus*), mumps and parainfluenza viruses (both *paramyxoviruses*). However, in contrast to the mumps and parainfluenza, measles and RSV do not possess neuraminidase. All members of this family are enveloped, negative-stranded RNA viruses. The virus consists of eight proteins encoded within a 16 kb genome. The measles F envelope glycoprotein mediates fusion after cell attachment; the measles Hemaglutinin (H) protein is involved in the attachment and entry of measles virus into cells via binding to the cell surface receptors, CD46 and signaling lymphocyte-activation molecule (SLAM, CD150)[341]. CD46 is involved in regulation of complement activation, is ubiquitously expressed on all nucleated cells and acts as a receptor for the measles Edmonston and Halle strains. SLAM is selectively expressed on some T and B cells and is used by both the Edmonston strain and wild-type measles strains that cannot use CD46 for cell entry[342]. Spontaneous tumor regression observed during wt measles infection can be explained by the relative overexpression of CD46 on human tumor cells and has resulted in the engineering of attenuated measles viruses derived from the Edmonston strain as selective oncolytic agents[53,341]. The high prevalence of preexisting anti-measles immunity as a result of world wide vaccination programs with live attenuated MMR (Measles, Mumps and Rubella) vaccine hampers the clinical use of oncolytic measles strains. However, from a safety perspective the use of oncolytic measles strains shows great potential as reversion of attenuated measles strains to pathogenic phenotypes has never been observed and population immunity provides the best protection from measles vector spread from patient to medical personal and close relatives[341]. The F and H proteins are the primary targets for the host immune response and induce a neutralizing antibody and cytotoxic T-cell response. The H protein of wild-type, but not vaccine strain Measles, activates TLR2 and stimulates production of IL-6[343]. Humoral and cellular immunity is mediated by induction of the Th1 and Th2 cytokines, IFN-γ and IL-4[344]. Measles virus clearance and long-lasting immunity is mediated predominantly by CTLs, as patients with agamma-globulinemia recover normally. However, in patients with an impaired cell-mediated immune response Measles pneumonia, although rare, is often fatal. Measles disease typically is more severe in adults and very young children (<5 yrs of age). The P gene of wild-type Measles virus encodes P/V/C proteins and antagonizes the IFN response. Phosphopolymerase (P) protein is a basic component of viral RNA polymerase, whereas the C and V proteins are non-structural accessory proteins[345]. Suppression of

immunity by MV can result in an increased susceptibility for secondary bacterial infectionPhosphopolymerase (P) protein is a basic component of viral RNA polymerase, whereas the C and V proteins are non-structural accessory proteins[312]. Suppression of immunity by MV can result in an increased susceptibility for secondary bacterial infection

New Castle Disease Virus

Newcastle disease virus (NDV) is a member of the Avulavirus genus in the Paramyxoviridae family, which has been shown to infect a number of avian species. NDV has a single-stranded negative sense RNA genome, which consists of six genes[346] and does not undergo recombination with the host genome or with other viruses[347]. NDV has a lipid–glycoprotein envelope and its spikes come from the hemagglutinin-neuraminidase (HN) and the fusion (F) genes. Attachment is mediated through the sialic acid cell receptor(s) and results in fusion with the cell membrane[346]. In response to NDV, IFNα and TNFα are released by peripheral blood mononuclear cells[348]. NDV induces a strong neutralizing antibody response and CTL response against HN and F protein complexes, which limit spread of oncolytic NDV strains to other tumor cells within the host[346].

Vesicular stomatitis virus

Vesicular stomatitis virus (VSV) infection of normal cells induces a potent type 1 interferon, which blocks viral replication. In contrast, many tumor cells have defects in their IFN response, allowing for selective lysis of tumors and the associated priming of T cells against tumor-associated antigens[349,350].

VIRAL EVASION MECHANISMS

Viral evasion mechanisms can be grossly divided into four categories: 1. Strategies to inhibit the innate immune response; 2. Strategies to evade recognition by the humoral immunity: These are mostly used by smaller viruses with a limited capacity to harbor genes, such as the picornaviruses, myxoviruses and retroviruses, which depend on the continuous modification of their viral envelope glycoproteins to prevent recognition of immunodominant epitopes by the immune system; 3. Interference with the processes of the cellular immune response. This is the main strategy applied by DNA viruses, including the poxvirus, herpesviruses and adenoviruses, which have a variety of mechanisms to prevent presentation by APCs to immune cells and use this to increase their chances for viral replication and shedding; and 4. Interference with immune effector functions, for example through expression of certain inhibiting cytokines or prevention of apoptosis[351].

Evasion of the innate immune response

The innate immune response is initiated when Toll-like receptors TLR3, TLR7/8, and TLR9 recognize their respective endosomal patterns associated with the viral genome, ie. dsRNA, ssRNA and CpG DNA, or when the RNA helicases, RIG-1 and Mda-5, recognize foreign cytoplasmic RNA molecules, which results through a series of intermediates in the production of type I interferons. Interference with these pathways results in modulation of the innate immune response. Vaccinia virus (VACV) employs a variety of distinct pathways to evade the immune response and regulate virulence, including expression of a wide range of immune modulatory proteins, such as A39R and A41L. Two other proteins, expressed by VACV, A52R and A46R, interfere with the host defense by modulating TLR pathways. Whereas A52R blocks the activation of NF-κB by multiple TLRs, in particular TLR3, by binding to IRAK2 and TRAF6[352], A46R, which is expressed by VACV early during infection, contains a Toll-like–interleukin-1 resistance (TIR) domain and acts as a decoy protein by targeting myeloid differentiation factor 88 (MyD88), MyD88 adaptor-like, TIR domain-containing adaptor inducing IFN-β (TRIF) and the TRIF-related adaptor molecule, and inhibits downstream activation of NF-κB and MAP kinase[353]. A46R thus disrupts TRIF-induced interferon (IFN) regulatory factor 3 (IRF3) activation and induction of TRIF-dependent genes. The HCV serine protease NS3/4A inhibits activation of IRF-3 by cleaving TRIF and IPS-1 downstream of TLR3/TLR4 and the RNA helicases and blocks type I interferon production in response to dsRNA[354]. Most paramyxoviruses V-proteins can inhibit induction of IFN-β through direct interaction with Mda-5, thereby blocking dsRNA binding[355,356]. In addition, select paramyxovirus V proteins from the genus *Rubula virus* inhibit dsRNA mediated signaling by mimicking IRF3, and act as alternative substrates for TBK1/IKKe[357].

Immune evasion mechanisms operated by HSV-1 include 1) management of humoral immunity: Viral glycoprotein gC can bind to and inactivate the C3 component of complement[358], viral glycoproteins gE and gI form a receptor complex that binds to the Fc portion of IgG[359]; 2) resistance against type I interferons: Immediate early protein ICP0 antagonizes type I IFN-induced STAT1-

dependent repression of viral replication[360-362], blocks the nuclear accumulation of activated IRF-3, required for transcription of type I IFN genes[363], and is necessary for efficient reactivation from latency[362], and ICP34.5 mediates dephosphorylation of eIF2α[364], as described above. The HSV-1 ICP47 protein, encoded by α47, blocks TAP, preventing viral peptides from being transported to the ER and assembling with MHC class I molecules[365,366]. The natural tropism of HSV-1 for epithelial cells and neuronal cells makes it an outstanding vehicle to use as a vector for neuronal gene transfer[274]. Interestingly, mutations in ICP47 result in a less neurovirulent HSV-1 strain, whereas replication in epithelial cells occurs normally[365].

Evasion of the humoral immune response

All RNA viruses are subject to a relatively high mutation rate as a result of the lack of proofreading control mechanisms of RNA polymerase. Influenza virus type A uses these mutations, which may result in antigenic drift or antigenic shift, to its own advantage, to evade the humoral immune surveillance system and ensure survival. Antigenic drift occurs due to accumulation of random point mutations in viral genes, coding for immune dominant epitopes, presented at the cell surface, such as hemagglutinin and neuraminidase. Antigenic shift, a consequence of exchange of large RNA segments between viral chains, may result in considerable changes in surface proteins[367]. These cell surface modifications affect the pre-existing host humoral immunity and new encounters with the virus will result in a primary immune response.

Other viruses, such as herpes viruses and corona viruses, express IgG Fc binding proteins that inhibit IgG activity[368]: HSV-1 glycoproteins gE and gI form an IgG Fc receptor, which upon binding inhibits Fc-mediated immune functions, enabling the virus or infected cell to evade an antibody attack[359]. Herpes viruses, Vaccinia virus and HIV-l each have the capacity to interfere with complement, either by incorporation of cellular complement regulatory proteins into the virion envelope or cell membrane, or by expression of viral molecules that mimic functions of complement regulatory proteins[368]: HSV-1 expresses glycoprotein gC, which can bind and inactivate the C3 component of complement[358].

Modulation of the cellular immune response

Viruses have developed multiple mechanisms to interfere with the activation of CD8+ and CD4+ T-cells by MHC class I and II molecules, respectively. Interference can occur from the moment the virus penetrates the cell, during degradation into small peptides, during association with the MHC molecules, till the moment of transport to the cell membrane and presentation to T-cells. Proteasomal degradation of specific sequences within the viral proteins results in the formation of smaller peptides with specific epitopes, which can be recognized by cytotoxic lymphocytes (CTL). Minimal modifications in the viral genome, affecting just a single amino acid, can prevent the degradation of the viral protein into immunodominant CTL epitopes[369]. As mentioned above, herpes viruses can persist in a latent state for longer periods of time and reactivate at later moments. The EBV encoded nuclear antigen-1 (EBNA-1) is essential for viral latency and is expressed in infected B cells of healthy EBV carriers[370]. The Gly-Ala repeat (Gar) of EBNA-1 is responsible for the *in cis* inhibition of ubiquitin/proteasome dependent proteolysis and prevents presentation of CTL epitopes by MHC class I molecules[370-372], thus preventing efficient recognition and killing of EBNA-1 positive cells. In addition, translation of EBNA-1 mRNA from an alternative open reading frame (ORF), results in a 40.7 kDa strongly acidic protein with a glycine, glutamin and glutamic acid-rich repeat (GZr)[373], which was also shown to inhibit antigen processing. The latency-associated nuclear antigen-1 (LANA-1) from Kaposi Sarcoma herpesvirus (KSHV or HHV8), which contains an acidic protein domain with high resemblance to GZr[374,375] and ORF73, a protein from the murine gamma-herpesvirus 68, are functional equivalents of EBNA-1 and both inhibit antigen processing[376]. Phosphorylation of specific viral proteins, such as matrix protein pp65 (UL83) of human CMV or phosphorylation of threonin residues in the immediate early (IE) proteins can inhibit proteosomal degradation as well[377].

Endosomal and lysosomal proteins are enzymatically degraded into smaller peptides, which bind to MHC class II molecules. The MHC class I-associated peptides are produced in the proteasome by proteolytic degradation of cytosolic proteins and are transported to the ER. Here, the peptides are linked with transporter associated with antigen processing (TAP) to newly synthesized MHC class I molecules. Competitive inhibition with the peptid/TAP complex, such as by the herpes simplex virus immediate-early (IE) gene transcript ICP47[377] and BHV1 UL49.5 proteins[378], hinders transport of antigenic viral peptides to the ER and assembly with MHC class I molecules and prevents CD8+ T-cell recognition of infected cells[365].

APPENDIX B IMMUNE MODULATION AND INDUCTION OF TOLERANCE

The viral vectors that are being used in gene therapy are able to induce the full range of host innate and adaptive immune responses. Removal of redundant viral genes in order to create vacant areas for transgenes and make vectors less immunogenic, may have in fact reciprocally contributed to the immunogenicity of the constructs by removal of viral genes that modulate immune responses and viral evasion mechanisms.

Immune modulation

Systemic immune suppression in pre-clinical animal models and gene therapy trials is used ideally to prevent induction of or, less ideally, tamper an existing immune response. A temporary incapacitated immune system could possibly 1) prevent early clearance of vector; 2) gain time to allow complete clearance of viral capsids from the circulation; 3) induce tolerance for the transgene by preventing a cellular immune response and the appearance of neutralizing antibodies. Improved and new generations of immunosuppressive agents developed for induction or maintenance therapy in hematological and solid malignancies and transplantation are jeopardized by a potential increase in infectious complications. Linking the risk of a particular infection to the use of a specific immunosuppressive agent has proven complicated, as many of the agents are used serially or in conjunction with each other and often in combination with other prophylactic and preemptive treatment strategies. An overview of immune suppressive and immune modulatory agents is given below and summarized in Table III. However, the degree and duration of immune suppression required to avoid allograft rejection or used for treatment of malignancies is likely to be lower and shorter for immune modulation of gene therapy, and depends largely on the amounts of antigen presented (eg transduction of antigen-presenting cells, targeted tissue and route of administration), the nature of the antigen (neoantigen) and number of antigen-specific T cells. In the case of transplant rejection, prophylaxis of rejection is generally more successful than attempts to eradicate an existing adaptive immune response. However, in gene therapy this is often not possible due to preexisting immunity present in the normal population. Nevertheless, the widespread use of immune suppressive treatments provides the gene therapist with a vast array of immune modulatory agents, the choice of which depends on the intended effects and observed side effects.

Systemic immune suppression

Glucocorticoids are among the most commonly used transient immune suppressive agents. In the C57Bl/6 mouse model, using intravenous injections of $0{,}75x10^{11}$ E1-deleted human Ad5-derived replication-deficient vector particles encoding the highly immunogenic β-galactosidase (lacZ), it was demonstrated that pre-treatment with dexamethasone (DEX) was sufficient to significantly reduce most Ad-induced innate immune responses and dampen the adaptive immune response without affecting the efficacy of the Ad vector mediated gene transduction or levels of transgene expresssion[197]. Pre-emptive treatment with DEX resulted in a dose-dependent decrease in release of systemic cytokines, such as IL-6, IL-12, G-CSF, keratinocyte-derived cytokine (KC), monocyte chemoattractant protein 1 (MCP-1) and macrophage inflammatory protein 1β (MIB-1β), a decrease in RANTES at doses as low as 0.1 mg/kg and completely prevented Ad-induced acute thrombocytopenia, endothelial cell activation, pro-inflammatory gene induction, and leukocyte infiltration into transduced organs at a dose of 10 mg/kg. Similarly, mice treated with 0.5-4 mg methylprednisolone (MP) before intravenous injection of 10^{11} particles of E1/E3/E4-deleted $AdAT_4$ expressing human apolipoprotein A-I (ApoA-I), displayed reduced levels of inflammatory cytokines IL-6, MIB-1β, MIB-2, interferon-inducible protein-10 (IP-10), lipopolysaccharide-induced CXC chemokine (LIX) and KC and decreased thrombocytopenia and leukocyte infiltration[55]. In a small pilot study involving 5 patients with mesothelioma, safety and efficacy of intrapleural administration of $1.5x10^{13}$ replication-incompetent E1/E3 deleted Ad5-based vector particles carrying the HSV-TK suicide gene (H5.010RSV*tk*) and concurrent treatment with iv 60 or 125 mg MP, starting at 10 hours before gene transfer, every 6 hours for 3 days, was assessed[379]. The study showed that, although the short course of high-dose corticosteroids to patients receiving intrapleural gene therapy did not affect the efficacy of gene transfer to the tumor, in contrast to the animal studies, it showed that it only moderately affects the cellular response and could not prevent the development of a strong anti-Ad humoral immune response. Although some of the acute systemic inflammatory responses, such as fever and hypoxemia, were decreased in the MP-treated group, the overall vector-related toxicity was similar to the control group of patients receiving no corticosteroids, and consisted of liver enzyme elevation, mild-moderate anemia, fever after vector infusion and bullous exanthema surrounding the thoracic access site. Reversible mental-status changes

in 3 patients using high-dose MP required lowering of the dose from 125 mg to 60 mg per infusion, but the overall treatment with MP appeared safe, with no evidence of increased viral shedding, other organ toxicity or disseminated viral infections[379].

Clearance of Ad, AAV and LV vectors is crucially dependent on the induction of a CD8+ T-cell response. Current regimens used in animal studies to block this response are loosely based on the immunosuppressive regimens used in organ transplantation for two reasons: the first one being that the organ transplant regimens are typically developed to block or diminish the CTL responses, the second one being the vast experience obtained with these drugs in the clinical setting. Immunosuppressive regimens commonly used include either monotherapy with Cyclosporine A (CSA), methotrexate (MTX) and cyclophosphamide (CY), sirolimus or daclizumab to repress the CTL reponse by inhibition of IL-2 production[380], or combination therapy with Mycophenolate Mofetil (MMF) or anti-thymocyte globulin (ATG) or tacrolimus. CSA is a calcineurin inhibitor, which specifically and reversibly inhibits proliferation of T-lymphocytes, without suppressing hematopoiesis or affecting the function of phagocytic cells. It inhibits lymphokine production and release from activated T-cells. This inhibiton is however not absolute and virus-specific responses can occur even in stem cell transplant recipients (personal communication with Marco Schilham). Thus, a very strong anti-viral response will not be sufficiently repressed by the use of CSA alone. It was found that neither the frequency nor the spectrum of infections in organ transplant recipients was remarkably affected by the type of calcineurin inhibitory agents *per se*, the most commonly observed viral infectious complication being CMV[381]. Rather, susceptibility appeared more dependent on the presence or absence of additional immune suppressants.

Due to its structural similarity to folic acid, MTX functions as a folic acid antagonist and inhibits synthesis of nucleic acids and proliferation. The use of MTX in a gene therapy setting has been limited to use in combination with transfer of the MTX drug-resistance gene, dihydrofolate reductase (DHFR)[382-384] to confer myeloprotection to bone marrow cells. Gene modified hematopoietic stem cells, carrying the DHFR are resistant to subsequent treatment with MTX, whereas malignant cells in solid tumors or metastases are sensitive to the cytotoxic effects of MTX. Although patients with rheumatoid arthritis are commonly treated with immunosuppressive agents, reactivation of latent EBV in these patients is uniquely associated with treatment with MTX[385].

CY is a DNA-alkylating agent and blocks progression through the cell cycle. Although it can result in full bone marrow depression, most of its immunosuppressive effects are mediated via inhibition of B-cells, CD4+T-cells and to a lesser extent CD8+T-cells. It also might function by inhibiting suppressor cells. In a muscular dystrophy mouse model, different regimens of transient immune modulation were used to prevent anti-AAV1 vector directed responses[386]. Although a five day blockade of co-stimulation with CTLA4Ig or anti-CD40 antibodies (as described in the next section) was sufficient to totally abrogate the formation of anti-AAV1 antibodies and allow correction of muscular dystrophy in injected muscles, 'conventional' immunosuppressive treatment consisting of combinations of FK506, MMF and cyclosporine with or without Prednisone failed to inhibit formation of AAV1-specific neutralizing antibodies[386]. However, in a non-human primate model of hemophilia A, transient treatment with FK506 and MMF for up to 6 weeks, resulted in 2 out of 3 animals in effective prevention of formation of anti-AAV8 capsid antigens, and although upon withdrawal of the immune suppressive agents an increase in anti-AAV8 IgG was observed, none of the animals developed anti-hFIX antibodies[54]. A dose-related risk for (disseminated) Herpes Zoster and CMV infections is observed in SLE patients, a patient population already at a high risk of infections, when treated with CY, particularly if the immunosuppressive regimen is used in combination with high-dose glucocorticoids[387].

Inhibition of specific immune responses

Specific immune responses can be inhibited by immune signal blocking depleting (Alemtuzumab, Rituximab, Muronomab) or non-depleting antibodies (Daclizumab, Basiliximab), or by blockage of co-stimulation signals with, anti-ICOS, anti-CD80, anti-CD86, anti-CD40L, IDO and CTLA4-Ig or less specifically, but more effectively with anti-thymocyte globulin (ATG).

Depleting and non-depleting antibodies

Alemtuzumab (Campath-1H) is a humanized monoclonal antibody against CD52, expressed by B and T lymphocytes, monocytes/macrophages and natural killer cells, which is predominantly used for the treatment of CLL. Alemtuzumab gives long lasting, profound lymphocyte depletion, which is associated with an increased occurrence of CMV and BK infections in solid organ transplant recipients, when used to prevent transplant rejection, but not when used as induction therapy[388] and with increased

CMV[389] and Ad[390] infections in hematopoietic stem cell transplant recipients in comparison to similar immune suppressive regimens without Alemtuzumab. The risk for viral infections in pediatric hematopoietic stem cell transplantation patients appears not to be increased in comparison to regimens using anti-thymocyte globulin (ATG)[391]. The absence of anti-murine or anti-canine CD52 antibodies reflects a lack in acquisition of pre-clinical animal data and might explain reluctance of initiating human studies using Alemtuzumab as immune modulation in gene therapy.

Rituximab is a chimeric human/mouse monoclonal antibody against CD20, and can be used for long lasting (9-12 months) depletion of B-lymphocytes. The use of Rituximab in solid organ transplant recipients was not associated with increased risks for CMV or BK infections[392], but rituximab treatment for post-transplant lymphoproliferative disorders (PTLD) has been associated with occasional fatal CMV reactivation[393] and with an increased risk for CMV in NHL patients receiving autologous stem cell transplantations[394] and both increased risks for CMV and HBV and reactivation of VZV in lymphoma patients receiving chemotherapy, but no stem cell transplantation[395]. Treatment of a rhesus monkey, that developed neutralizing antibodies against human coagulation factor IX in a non-human primate model of gene therapy for hemophilia B, with two doses of rituximab and daily doses of cyclosporine for four weeks, demonstrated that this regimen was sufficient to eradicate anti-hFIX antibodies, which appeared after treatment with a self-complementary AAV-based vector with a codon-optimized gene for human coagulation factor IX[396].

The murine monoclonal antibody Muromonab (OKT3) is directed against the T-cell surface molecule CD3 antigen, which is responsible for signal transduction. Treatment with muromonab results in short-term depletion of T-cells and recovery of T-cells occurs within a week after withdrawal of treatment. Muromonab is most commonly used to prevent (renal) transplant rejection and in the treatment of GvHD. In comparison to treatment with high dose MP (2 mg/kg) alone for treatment of GvHD following allogeneic hematopoietic stem cell transplantationin, treatment regiments consisting of high dose MP in conjunction with Muronomab resulted in considerably less viral infectious complications, with an observed decrease in CMV, EBV and HHV6, but a slight increase in incidence of Parvovirus B19 and adenovirus infections[397]. In a mouse study investigating the effects of the immune response on Ad vector clearance, a hamster-anti-mouse CD3 monoclonal antibody was used to deplete T-cells and prevent immune responses against the LacZ transgene[398]. A first generation E1-deleted Ad5 vector coding for the LacZ gene under control of the murine pancreatic amylase promoter (Ad5-Amy*LacZ*) was directly injected iv in C57Bl/6 mice. Both immune deficient NOD-SCID mice and C57Bl/6 mice treated with anti-CD3 antibody displayed lack of liver inflammation and produced stable lacZ expression in over 80% of hepatocytes at 3 weeks in contrast to immune competent control animals, which developed an inflammatory response and lost transgene expression[398].

Treatment with anti-thymocyte globulin (ATG) in humans results in a profound and long-lasting depletion of leukocytes. ATG is nonspecific and leukocytes, as well as certain subsets of NK cells, DC cells and monocytes are depleted from peripheral blood and lymphoid tissues through complement-mediated lysis and induction of apoptosis[399]. In solid organ transplant, the use of ATG is associated with an increased risk for CMV, EBV, and BK polyomavirus infections, in comparison with treatment with interleukin (IL)-2a receptor antagonists[400]. However, the risk for adenovirus infections appears to be lower in bone marrow transplant recipients treated with ATG than patients receiving Alemtuzumab[390]. In general, the risk for EBV and CMV infections appears to be related to the level and duration of T-cell depletion, which in turn depends on the dose of ATG used[401], the manufacturer (Fresenius versus Sangstat-Genzyme)[402], and the animal source (horse versus rabbit)[403]. In a mouse model of gene therapy for hemophilia A, different non-myeloablative regimens were tested, consisting of 5 Gy TBI with or without anti-murine thymocyte serum (ATS) and a combinations of BU and FLU with or without CY, CTLA4Ig, anti-CD40Ig and ATS[404]. After myelosuppression, mice were transplanted with HSCs carrying the porcine fVIII gene. It was demonstrated that addition of ATS to either the TBI or BU regimen was sufficient to achieve adequate and sustained levels of porcine fVIII, even if the mice were pre-immunized with human fVIII[404]. In a X-linked muscular dystrophy (*cxmd*) canine model, dogs were intramuscularly injected with rAAV6-CMV-h-μ-dys, carrying a human dystrophin gene[405]. Immune suppressive treatment consisting of CsA and MMF was not sufficient to suppress local T-cell responses to AAV in injected muscles. In contrast, triple immunosuppressive treatment with CsA, MMF and ATG allowed long-term expression of both human dystrophin and canine micro-dystrophin in *cxmd* dogs[405].

Daclizumab and Basiliximab are non-depleting antibodies, directed against the IL-2 receptor alpha chain (CD25) of activated T-lymphocytes and prevent T-cell proliferation. Their actions last up to 3 months and infectious complications associated with these agents are largely dependent on the duration of treatment. Induction or short-term therapy generally results in a decreased risk of viral infections, whereas maintenance or long-term therapy in transplant patients is associated with an increased risk of CMV infections and death[406]. Rhesus macaques were used to study the effects of immune modulation on transgene expression after direct infusion of AAV2-fIX into the hepatic artery[407]. As many hemophilia and renal transplant patients are HCV+, the immune suppressive drugs chosen for this study were based on a regimen, commonly used in renal transplantation, which had been previously shown to offer an excellent long-term safety profile in HCV+ patients[408]. They compared the effects of combination treatment of MMF and sirolimus with or without daclizumab. It was previously shown that the immunologic tolerance to the transgene, achieved in hepatic gene transfer, is mediated by CD4+CD25+FoxP3+ regulatory T-cells[409] and therefore immune suppressive regimens should be designed to block the function of CTLs, while maintaining induction of Tregs. Here it was shown that a regimen consisting of MMF and sirolimus resulted in low level transgene expression and low levels of non-neutralizing anti-fIX antibodies, whereas addition of daclizumab to the regimen resulted in the formation of high levels of inhibitory antibodies directed against fIX and complete loss of transgene expression[407]. The use of 2 or 3 drug regimen did not affect the transduction efficiency, but it was found that the addition of daclizumab resulted in an increased B-cell response to the AAV capsid and a marked reduction of CD4+CD25+FoxP3+ regulatory T-cells.Daclizumab and Basiliximab are non-depleting antibodies, directed against the IL-2 receptor alpha chain (CD25) of activated T-lymphocytes and prevent T-cell proliferation. Their actions last up to 3 months and infectious complications associated with these agents are largely dependent on the duration of treatment. Induction or short-term therapy generally results in a decreased risk of viral infections, whereas maintenance or long-term therapy in transplant patients is associated with an increased risk of CMV infections and death[354]. Rhesus macaques were used to study the effects of immune modulation on transgene expression after direct infusion of AAV2-fIX into the hepatic artery[355]. As many hemophilia and renal transplant patients are HCV+, the immune suppressive drugs chosen for this study were based on a regimen, commonly used in renal transplantation, which had been previously shown to offer an excellent long-term safety profile in HCV+ patients[356]. They compared the effects of combination treatment of MMF and sirolimus with or without daclizumab. It was previously shown that the immunologic tolerance to the transgene, achieved in hepatic gene transfer, is mediated by CD4+CD25+FoxP3+ regulatory T-cells[357] and therefore immune suppressive regimens should be designed to block the function of CTLs, while maintaining induction of Tregs. Here it was shown that a regimen consisting of MMF and sirolimus resulted in low level transgene expression and low levels of non-neutralizing anti-fIX antibodies, whereas addition of daclizumab to the regimen resulted in the formation of high levels of inhibitory antibodies directed against fIX and complete loss of transgene expression[355]. The use of 2 or 3 drug regimen did not affect the transduction efficiency, but it was found that the addition of daclizumab resulted in an increased B-cell response to the AAV capsid and a marked reduction of CD4+CD25+FoxP3+ regulatory T-cells.

Blockade of co-stimulation

An alternative approach to inhibition of specific immune responses is blockade of co-stimulatory signals delivered by the B7-CD28 or CD40-CD40L (CD154) pathways. CD28, CTLA4 and ICOS all belong to the CD28 receptor superfamily[410].

Cytotoxic T-lymphocyte–associated antigen 4 (CTLA4) is a cell surface protein present on T-lymphocytes. CTLA4 is endogenously responsible for regulating T-lymphocyte activation and is upregulated after T-lymphocyte activation. CTLA4 competes with CD28 and binds to CD80/CD86 (B7) with greater avidity than CD28, thereby blocking CD28 binding. This results in the production of IFN-γ and upregulation of indoleamine 2,3 dioxygenase (IDO)[386] and down-regulation of the activated T-lymphocytes and the immune response. Abatacept is a recombinant fusion protein, which consists of the extracellular domain of CTLA4 and the Fc domain of IgG (CTLA4Ig), and is mainly used in the treatment of rheumatoid arthritis patients with inadequate responses to MTX or TNFα-inhibitors[411]. The use of Abatacept in these patients is not associated with an increased risk in viral infections and is generally well tolerated[412]. In *in vivo* Ad vector gene therapy animal models, CTLA4Ig is commonly used as an immune modulatory agent, either as direct intraperitoneal or intramuscular injections[386,413-417] or inbedded in the vector contruct[413,418]. Whereas the

former is used for gene therapy of a wide range of disease models (see Table II), ranging from hemophilia to muscular dystrophy, the latter is predominantly used as gene therapy to prevent solid organ (xeno) transplant rejection in animals[418-423]. In contrast to CTLA4Ig, which blocks both CTLA4 and CD28 mediated co-stimulation, anti-CD28 antibodies may prove more specific. CTLA4Ig reduces T-cell co-stimulation through competition with CD28, but also prevents CTLA4 from transmitting negative signals, which are important for the development of regulatory T-cells and are required for induction of tolerance[424]. Anti-CD28 antibodies were shown to prolong allograft survival in rats[425], in particular when combined with CD40Ig[426]. Although humanized anti-CD28 antibodies have been developed[427], currently effects of anti-CD28 antibodies on risk for specific infections in a human setting are unknown.

CD40Ig is a secretable fusion protein, which blocks the CD40-CD40L pathway. Similar to CTLA4Ig and anti-CD28 antibodies, CD40Ig is mainly used in animal gene transfer models to facilitate and induce tolerance to allografts[428,429]. CD40Ig expression is most commonly achieved by gene transfer using adenoviral vectors and is often used in combination with another adenoviral vector carrying the CTLA4Ig gene[430-433]. There are no reports on the use of CD40Ig in a clinical setting.

The effects of transient inhibition of co-stimulation using anti-human CD40 with or without anti-human CD86 chimeric antibodies were tested in a non-human primate model[434]. Administration of anti-CD40 and anti-CD86 antibodies delayed or blocked the development of neutralizing antibodies against the adenoviral vector and prevented infiltration of CD8+ cells in the liver, resulting in prolonged persistence of transduced cells and efficient re-administration of adenoviral vector. In agreement with data from other studies involving blockade of co-stimulation, administration of both anti-CD40 and anti-CD86 resulted in more efficient treatment than with anti-CD40 antibodies alone, demonstrating that inhibition of multiple pathways of co-stimulation leads to enhanced immune suppression[434].

Although administration of anti-CD40L antibody for 2 weeks, in order to block CD40-CD40L interactions, was shown to prolong expression of β-glucuronidase after intravenous delivery or brain injections of E1-deleted Ad5.βgluc in a mouse model of MPS type VII[435].

Inducible co-stimulator (ICOS) is expressed by memory T cells, which undergo proliferation independent of B7-CD28 or CD40-CD40L signaling. The ICOS ligand (ICOSL) is constitutively expressed on B cells and is inducible on monocytes, dendritic cells, fibroblasts, and endothelial cells. Blockade of ICOS-ICOSL interactions blocks not only T-helper (Th) type 2 signaling, but also Th1-dependent responses. Anti-ICOS antibodies were used in combination with AdCTLA4Ig to prevent cardiac allograft rejection in rats[418]. Although treatment with AdCTLA4Ig alone could prevent acute heart rejection, signs of chronic rejection, including myocyte and vessel injury, as well as transplant arteriosclerosis, were found 100 days after transplantation. Addition of mouse-anti-rat ICOS antibodies resulted in a dramatic reduction of affected vessels and mononuclear cell infiltration and prevention of chronic rejection. However, anti-ICOS antibodies could not prevent hyperacute rejection. Combination treatment with either AdCTLA4Ig[418] or CD40Ig[436] could prevent rejection, underlining the importance of inhibiting multiple pathways for effective co-stimulation blockade resulting in induction of tolerance and prevention of rejection. Recently, the immune modulatory effects of a fully human monoclonal antibody against human ICOS (JTA-009) were studied in a graft-versus-host model, where human peripheral blood mononuclear cells were engrafted in SCID mice[437]. JTA-009 was shown to significantly prolong mouse survival. There are no available data of the use of anti-ICOS antibody in a clinical setting.

Tryptophan is an amino acid essential for protein synthesis and vital metabolic functions. Two enzymes are involved in the catabolism of tryptophan along the kynurenine pathway, Tryptophan 2,3-dioxygenase (TDO), which is mainly expressed in the liver and Indoleamine 2,3-dioxygenase (IDO), which is present in many tissues and is induced by inflammatory signals. IDO catalyzes the rate-limiting step in tryptophan degradation and has been shown to play a critical role in the regulation of immune tolerance to foreign antigens within the tissue microenvironment[438]. Where IDO itself suppresses T-cell proliferation, the formed tryptophan metabolites induce T-cell apoptosis[439]. IDO also mediates the tolerogenic effects of CTLA4Ig and CD40Ig by increasing the levels of circulating regulatory T-cells[440] and by specifically inhibiting the generation and function of allo-specific central memory CD8+ T cells, while effector memory CD8+ T-cell function remains unaffected[439]. The immunosuppressive effects and mechanisms of induction of graft tolerance by IDO have been studied by IDO induction with IFN-γ or local overexpression after gene transfer in allo- or xenografts in animal transplantation models[441,442], but not in a clinical setting.

Use of anti-inflammatory cytokines

Immune modulation can also be achieved by interfering with the inflammatory cascades using either anti-inflammatory cytokines, such as IL-10 or TGFβ, or by using specific antibodies directed against inflammatory cytokines, including anti-IFNα and anti-IFNβ[189]. Adenoviral vectors induce both innate and adaptive immune responses through the induction of high levels of type I IFNs[189,443]. Administration of neutralizing antibodies against mouse IFN-α and IFN-β, 6 hours before infusion of Ad-LacZ vector and again 5 days after infection to healthy C57Bl/6, resulted in a diminished immune response against the vector, more stable transgene expression and reduction of inflammation[189].

Proteasome inhibitors

Proteasome inhibitors, such as Bortezomib, are small molecules that are able to specifically inhibit the activity of the proteasome, resulting in an increase of ubiquitinated proteins, increased intracellular reactive oxygen species[444] and a decrease in presentation of MHC class I peptide complexes[445]. Bortezomib is predominantly used in the treatment of refractory multiple myeloma and may result in transient peripheral neuropathy, transfusion-dependent thrombocytopenia and neutropenia with an increased risk for fungal, viral and bacterial infections. The use of Bortezomib is associated with the reactivation of Herpes Zoster (VZV)[446], but may result in lethal herpes infections by CMV or HSV, particularly in cases where the agent is used in combination with Dexamethasone. In mice, Bortezomib was shown to enhance the susceptibility to viral infections with the lymphocytic choriomeningitis virus (LCMV), due to a decreased cytotoxic T-cell response[447]. The administration of bortezomib to mice after infection with LCMV did not influence the cytotoxic T cell response, suggesting that Bortezomib is effective during the initial priming of naive T cells[447]. More recently, Bortezomib was shown to facilitate AAV transduction both *in vitro* and *in vivo* and for multiple serotypes and cell types[448,449]. Possible mechanisms include changes in AAV intracellular processing due to modulation of the ubiquitin-proteasome system and decreased capsid-derived peptide epitope presentation on MHC class I[449].

Induction of tolerance

Immunological tolerance is the process in which the immune system is responsive to foreign antigens, while being tolerant to self-antigens. In gene therapy, the goal is to achieve tolerance towards exogenous neo-antigens, characterized by an antigen-specific nonreactivity of the immune system. Tolerance can be established centrally at the site of primary lymphocyte development (thymus and bone marrow), or in the peripheral lymphoid tissues, where antigen recognition and processing occur. Central tolerance is achieved through a process of positive and negative selection, whereas peripheral tolerance is brought about through clonal deletion of effector T-cells (Teff), induction of unresponsiveness (anergy), ignorance and active suppression by regulatory T-cells (Treg).

Regulatory T-cells

CD4+/CD25+/FoxP3 regulatory T-cells are stimulated by IL-10 and TGFβ, and induce tolerance by suppression of ongoing immune responses, through cell contact-dependent suppression of IL-2 expression, *in vivo* secretion of suppressive cytokines and even killing effector T-cells or APCs[450]. Activation of Tregs is used for induction of tolerance in transplant recipients, but can also be used to modulate immune responses against transgenes and genetically modified cells[407]. Regimens containing sirolimus are of great interest here, as the drug has been shown to promote induction of regulatory T cells[451]. Other immune modulating agents positively affecting Tregs are glucocorticoids, mTOR inhibitors, certain depleting antibodies, including anti-CD3 mAb (OKT3) and Alemtuzumab, CTLA4Ig and IvIg (reviewed by Arruda *et al*)[452]. In contrast, the use of immune modulating agents that negatively affect levels of immune response suppressing Tregs can result in an increased immune response and loss of transgene expression. Examples are cyclophosphamide, the calineurin inhibitors cyclosporine and tacrolimus, horse ATG and the monoclonal antibodies daclizumab (anti-CD25) and basilixumab, which binds to activated T-cells. The importance of the Tregs for tolerance towards viral vectors and transgenes was demonstrated by a study in non-human primates, in which animals were treated with an AAV2 vector expressing human fIX and simultaneously received immune suppression with MMF, rapamycin with or without daclizumab[407]. Animals treated with MMF and rapamycin displayed decreased anti-AAV2 antibodies, whereas animals treated with MMF, rapamycin and daclizumab displayed decreased levels of Tregs and not only had increased antibody responses against the viral vector, but also developed neutralizing antibodies against the fIX transgene.

Hepatic gene transfer

Daily, large amounts of blood flow through the liver and many foreign antigens are presented to hepatocytes, he-

patic stellate cells, Kupffer cells, dendritic cells, sinusoidal endothelial cells and lymphocytes. These cells all play a different role in immune modulation and induction of tolerance. Hepatocytes express a range of immunomodulatory markers, including MHC-1, CD-1 and ICAM-1, they lack constitutive expression of co-stimulatory molecules[453]. Kupffer cells (KC) are scavengers of pathogens, including viral particles and express MHC-II molecules, ICAM-1, CD80 and CD86. Phagocytosis of pathogens by KC results in immediate production and release of proinflammatory cytokines, but in response to stimulation with lipopolysaccharide, KC secrete IL-10, TGFβ and prostanoids, known to promote tolerance. Hepatic DCs present foreign antigens to peripheral lymphoid tissues, but are also known to be important for tolerance following transplantation. Liver sinusoidal endothelial cells also have an antigen presenting function, but induce antigen-specific tolerance rather than immunity.

Ad vectors are rapidly phagocytosed by KCs and induce activation of IL-1β, release of proinflammatory cytokines and a potent anti-viral IFN type I immune response. KC presentation of Ad antigens to CD4+ and CD8+ cells then result in an adaptive immune response. KC depletion results in increased bioavailability of vector DNA and transgene expression in the liver[454]. The use of a tightly regulated hepatocyte-specific promoter can further reduce transgene expression in APCs. AAV vectors do not induce a potent immune response in the liver and are therefore preferred vectors for liver-targeting. However, a preexisting neutralizing antibody response against AAV or the transgene can complicate optimal liver transduction. *In vivo* liver-directed gene therapy with LV vectors induces an adaptive immune response against the transgene product as the result of an early type I IFN response to the RNA genome of the enveloped virus[455] and activation of T-cells due to transduction of APCs[456]. Here, the use of a hepatocyte-specific promoter alone is not sufficient to prevent this immune response[321].

After intramuscular injection of an AAV2 vector expressing the blood coagulation factor IX (fIX), in both normal immunocompetent mice[457] and dog models of hemophilia B[458] a rapid humoral immune responses against the fIX transgene was observed, preventing fIX from reaching therapeutic levels. In contrast, liver-directed gene transfer was shown to overcome neutralizing antibody formation by creating a tolerogenic environment for AAV2[409]. Thus, liver-targeting can also be used to treat non-liver diseases, where systemic delivery of a protein is needed, such as metabolic disorders and lysosomal storage diseases[453]. Tolerance induction through liver-specific gene targeting requires the use of a minimally immunogenic vector, the presence of sufficiently high levels of therapeutic transgene expression in hepatocytes[321], and the absence of transgene expression in APCs. The latter can be achieved through the use of tightly regulated hepatocyte-specific promoters with or without a miRNA-regulated transcript[456]. Under these circumstances full tolerance can be achieved, as evident from the presence of transgenic protein-specific CD4+ T-cells and the absence of neutralizing antibody formation and CTL responses, even after repeated challenge. The role of Tregs in induction of tolerance to the transgene product after *in vivo* hepatic gene transfer is clearly shown by Cao *et al*[459]. Induction of Tregs against human fIX was observed as early as two weeks after hepatic AAV-mediated gene transfer in normal mice. Depletion of Tregs resulted in antibody formation against fIX, whereas adoptive transfer of these cells to Treg negative mice treated resulted in suppression of antibody formation to fIX[459]. Importantly, multiple studies have shown that immune tolerance to a foreign protein is maintained in other organs if that protein is expressed first in the liver. For example, after supplementary gene transfer with an AAV2 or E1/E3 deleted Ad vector to muscle after initial hepatic transduction with AAV2-fIX tolerance to fIX was maintained[460].

Muscle gene transfer

Immune tolerance to transgenic proteins can also be achieved following intramuscular gene transfer[461,462]. High expression of the transgene was shown to be critical for tolerance induction following intramuscular AAV injection[463]. The mechanism however appears to be distinct from immune tolerance induced by hepatic gene transfer and not mediated by Tregs. No significant increase in Tregs was seen after AAV1-fIX intramuscular gene transfer and tolerance induction to fIX. Adoptive transfer of splenocytes from fIX-tolerant mice was not able to suppress anti-hfIX immunity in recipient mice and depletion of Tregs in tolerized mice did not result in loss of tolerance to fIX[462]. Thus, it appears that T-cell anergy plays a major role in achieving peripheral tolerance after intramuscular gene transfer.

Prevention of binding of Ad to clotting factors

High-affinity interaction between coagulation factor X (fX) and Ad hexons for a number of serotypes, including Ad5, mediates Ad uptake into hepatocytes after intravenous Ad injection into mice[464-466]. Preinjection of snake venom factor X-binding protein (fX-bp) reduces hepatocyte transduction and thus prolongs the circulation time

of the fiber-chimeric Ad5/35 vector in peripheral blood[467].

Prevention of immune detection

Stealth-mechanisms

Methods or "stealth mechanisms" employed by the viruses to evade recognition by the immune system, as described by Zaldumbide *et al*[468], and methods to engineer non-immunogenic or immune-escaping viral vectors are currently being explored to enable the use of common serotypes to which the majority of the population already has pre-exixting immunity, to enable the use of specific characteristics (eg tropism) of otherwise immunogenic serotypes and to allow the reuse and readministration of vector, when a single treatment does not result in sufficient responses.

Adenoviral vectors display roughly 18000 amino groups on their cell surface. Modification of these amino groups with molecules, such as activated esters or large polysaccharides[469], can be used to efficiently modify large areas of the capsid surface. One of the most common methods to shield viruses is PEG-ylation of vectors. Polyethylene glycol (PEG) is an uncharged, nontoxic, hydrophilic, non-immunogenic polymer, which can be covalently attached to the Ad surface[470]. Different types of PEG with respect to molecular weight, branches, or active groups, affect the level of vector shielding and tropism (F. Kreppel, ESGCT 2009), but maintain the viral titer of the Ad vectors after storage and positively influence the stability at various temperatures[471-473]. When administered intravenously, the halflife of conventional Ad is less than 2 minutes, and most of the vector is accumulated in the liver. PEGylation of Ad vectors results in a four-fold decreased clearance and detargeting of the liver[474]. The amount of PEG coating on the surface of the Ad affects the characteristics of the vectors as well: the higher the modification ratio (ie PEG-coated surface area), and the larger the PEG molecules, the more efficient the shielding of immunogenic epitopes[475] and the lower transduction of liver[470]. Decreased immune responses against PEG-Ad are the result of both reduced innate IL-6 responses, which parallel a similar reduction in vector uptake by macrophages[476] and reduced humoral and cytotoxic T-cell responses[471].

Büning and collegues generated a capsid library by codon randomization at five known immunogenic sites[152,477] of the structural proteins ORF (*cap*) of AAV-2 and screened for viral mutants with an antibody escaping phenotype[242]. They found that the replacement of large hydrophobic amino acids at immunogenic sites by smaller hydrophilic amino acids increased the likeliness of tolerance towards the AAV-2 capsid. In addition, substitution of a limited number of residues was found to result in immune-escaping mutants, which retained the packaging ability, infectivity and tropism of the original AAV2 serotype, whereas major capsid modifications result in dramatically altered tropism. Noteworthy however, despite increased antibody evasion, all mutants were fully neutralized at high concentrations of human serum, indicating that immune evasion *in vivo* by stealth mechanisms remains difficult. Another method to obtain stealth phenotype may be by swapping entire antigenic domains between viral serotypes[150,478,479].

Prevention of early vector clearance

Complement depletion

It was previously shown that rodent plasma can inhibit cell transduction by replication-conditional (oncolytic) HSV, replication-defective HSV, and adenovirus vectors[68]. *In vitro* depletion of complement with mild heat treatment or *in vivo* depletion by treatment of athymic rats with cobra venom factor (CVF) partially reverses this effect. CVF is a structural and functional analog of the C3 component of complement and through continuous activation of C3 leads to depletion of complement[480]. CVF has been successfully used in animal models of xenotransplantation in order to prevent the hyperacute rejection reaction caused by natural antibodies and complement[481]. New human C3/CVF hybrids have been developed for therapeutic complement depletion[482]. In animal studies it was shown that depletion of complement allowed enhanced initial infection of tumor cells by the intravascular HSV vector hrR3 and this effect was even more pronounced if the treatment was followed by treatment with Cyclophosphamide to inhibit both innate and neutralizing humoral antiviral responses[68].

AAV capsid binding to C3 complement proteins enhances AAV uptake into macrophages, macrophage activation and induction of inflammatory cytokines and induction of neutralizing antibodies[223]. Complement receptor 1/2 and C3-deficient mice were shown to display impaired humoral immunity against AAV2 vectors and complement depletion may therefore delay the AAV-induced antibody development.

APPENDIX C LESSONS FROM THE PAST

Viral infections in patients with impaired immunity

Immune deficiencies

The primary severe combined immune deficiencies (SCID) can be divided into two separate, but closely linked groups, i.e. the immune deficiencies predominantly involving B cells and hence immunoglobulin and antibody production, and the immune deficiencies mainly affecting T cells and therefore cell-mediated immunity. Sensitivity for specific viral infections is strongly correlated to the cytopathogenic effects caused by a certain type of virus. Lytic viruses, such as enteroviruses are generally encountered by antibody responses, whereas viruses creeping from cell-to-cell, such as herpes-, myxo- and paramyxoviruses encounter cell-mediated immunity. The secondary or acquired immune deficiencies can be subdivided into secondary due to other diseases (eg AIDS), or secondary due to disease-related treatments (eg myelo- and/or immunosuppressive treatment for transplantation patients).

Adenovirus infections are traditionally associated with mild respiratory, ocular, or gastrointestinal disease, occurring predominantly in children and U.S. military recruits as endemic infections or during outbreaks[15,176]. During childhood these infections go often unnoticed, are usually self-limiting and result in serotype-specific immunity. The most prevalent serotypes belong to species A, B and C, i.e. 1, 2, 3, 5, 7 and 41 among civilians and 4, 3 and 21 among militairy personnel[176,483]. Typically, the patients infected with serotypes 5 or 21 had a higher risk of developing severe Ad disease[483]. In immunocompromised patients however, adenovirus infection can be more severe, give high morbidity and even result in mortality. Both primary infections or reactivation of latent Ad can occur. The serotypes described above account for approximately 50% of the infections in SCID patients, and other serotypes isolated from SCID patients include serotypes 11, 31, 34 and 35[484] (see Table XI and Table XII). Coinfection with more than one Ad serotype is also more common in immunocompromised patients than in immunocompetent patients[483]. Importantly, these coinfections may be not only from different serotypes, but also from different species[485]. Most of the serotypes of Ad species D have been isolated from AIDS patients, whereas species D is rarely found in the normal population and does not cause illness (personal communication with Marco Schilham). It is conceivable that long-term Ad coinfections in AIDS patients with multiple strains may provide the opportunity for mutations within a strain and allow recombination between serotypes. T-cell-mediated immunity is important for recovery after an acute Ad infection. Consequently, immunocompromised patients, lacking effective cellular immunity are at higher risk of Ad infection. However, the humoral response against AdV is also very important for controlling the infection, leaving in particular pediatric hematopoietic stem cell transplantation recipients prone to infection and to a lesser extent adult hematopoietic stem cell recipients (personal communication with Marco Schilham)[176]. Adenovirus infections occur in upto 3-47% of patients following stem cell transplantation (SCT), with an associated mortality of up to 70%[486,487].

Transplantation patients

The most commonly used immunosuppressive agents used to prevent rejection after kidney transplantation include (a combination of) corticosteroids, azathioprine, calcineurin inhibitors (CsA, tacrolimus), mTOR inhibitors (sirolimus) and mycophenolate mofetil. Immunosuppressive treatment often renders the patients susceptible to a wide range of viral infections, as the result of reactivation of latent viruses or primary infections with members of the herpes virus family: HSV-1, VZV, EBV, CMV, HHV-6, HHV-7, HHV-8/KSHV; community-acquired respiratory viruses: Ad, RSV, influenza, parainfluenza, Metapneumovirus; Parvovirus B19; donor-derived viruses: West Nile virus (in endemic areas only), Rabies, Hepatitis B and C, HPV, Polyomavirus BK/JC, HIV and SARS (coronavirus)[488]. Viral infections after transplantation follow a general pattern, with in the first two months posttransplant most commonly acute infections with Herpes viruses (HSV, EBV, VZV and CMV) and donor-derived viruses (HBV, HCV), and after 2-6 months acute community acquired infections (influenza and RSV) and chronic infections with CMV, EBV, HCV, HBV, HPV and BK[488]. Different immunosuppressive drugs have been associated with a susceptibility to distinct viral infections, such as steroids with HBV and HCV, T-cell depleting antibodies with reactivation of herpes viruses and HIV, tacrolimus with polyoma-related nephropathy[489], anti-thymocyte globulin and mycophenolate mofetil (MMF) with CMV[490]. Mycophenolate mofetil (MMF) is the prodrug of mycophenolic acid (MPA) and is used as an immunosuppressive agent in kidney transplant recipients[491]. MPA is a potent inhibitor of inosine monophosphate dehydrogenase (IMP-DH), which results in a depletion of the intracellular GTP and dGTP in T and B-cells, preventing their proliferation

and suppressing the cell-mediated and humoral immune response. MPA has been shown to inhibit the replication of a number of viruses, including arena viruses, yellow fever virus, reovirus-1, parainfluenza-3 virus, Coxsackie B4 virus, Epstein-Barr virus, Hepatitis B virus and human immunodeficiency virus[492].

Recombination and shedding of live-attenuated vaccine viruses

From vaccination studies, it can be learned that

- adults are unlikely to transmit to other adults high vaccine vector titers in pre-immune young children may increase the risk of transmission to other young children or even adults
- shedding and secondary/tertiary transmission most often occur via direct contact with the site of vaccine inoculation
- the risks of transmission depend on other environmental factors including frequent contact with excreta from other young children and the level of immune competence
- transmission may occur due to breeches in precautionary measures despite proper instruction
- risks of recombination between recombinant viral vectors and related wild type viruses will be the highest in the pediatric pre-immune population
- the risk of shedding is likely affected by additional handling, such as modification of vectors, immune modulation, the route of administration; the delivered viral vector load (single dose or/re-administration)
- Immune suppression or modulation may affect the environmental risk by two mechanisms:
  - longer persistence of the vector increases the duration of interaction with wild type viruses and recombination
  - immune suppressed individuals are more susceptible to infections, thus increasing the chances of interaction with specific wild type viruses

Gene therapy vector vaccines

HSV vaccines

Both live-attenuated and replication defective anti-HSV vaccines have been developed, but the live-attenuated viral vaccines have many advantages over the replication incompetent vaccines. Being able to present almost all viral antigens, lacking only those required for attenuation, live-attenuated HSV vaccines stimulate both the humoral and cellular host immune system more effectively. However, they also harbor the potential risk for neurovirulence, reactivation from latency, recombination with wt virus and possible shedding, the instability of the genotype during production[258,493,494] (laboratory strains of mutant HSV strains are often more attenuated than clinical isolates with the same mutation[495]) and oncogenesis[58,154,496]. Not all of the modifications made to achieve a safe live-attenuated HSV vaccine strain can be easily translated to the human situation due to species and virus-specific differences: Deletion of TK from HSV-1 reduced murine acute lethality and reactivation, whereas TK-deficient HSV-2 could still cause disease in humans[58,497]; deletion of a portion of RR yielded an attenuated HSV-2 strain with protective immunity in an animal model, but was not developed for human use due to high neurovirulence[498]; strain NV1020 (formerly R7020), based on HSV-1 strain F, was created by a deletion from UL54 (ICP27) to the promoter region of ICP4[499,500], but was found to be too attenuated to provide protection in humans. Vaccine strain RAV 9395, derived from HSV-2 strain G, carries deletions in the UL55 and UL56 genes and a deletion of both copies of the γ34.5 gene and was found to be protective in guinea pigs, but has not been tested in a clinical setting[501]. Various replicative-defective HSV-based vectors have been developed to serve either as an anti-Herpes vaccine[502,503] or as a vaccine vector. HSV-1 mutants lacking either immediate-early gene ICP4 or ICP27 or early gene ICP8 were shown to be capable of inducing a T-cell response in BALB/c mice and protected mice from challenge with wt HSV-1[502]. However, lack of production of significant levels of late proteins, many of which elicit protective immune responses, results in an incomplete suboptimal immune reponse. The discontinuously replicating or disabled infectious single cycle (DISC) HSV vectors have a deletion of UL22, the late gene encoding gH, and can infect a noncomplementing cell, but resulting viral progeny cannot infect a secondary cell[503]. Both HSV-1[504] and HSV-2[505] DISC vaccines were protective in a guinea pig model upon challenge, but more importantly, unlike some replication competent HSV vectors[257], the HSV-2 DISC virus was shown to be avirulent in the nude athymic mouse model. Although in a randomized placebo controlled clinical trial treatment with a HSV-2 DISC vaccine was shown to be safe, no clinical benefit was achieved, whereas asymptomatic viral shedding was detected in 82% of the persons following completion of the vaccination[506]. Other clinical trials found the gH-deleted HSV vaccine safe and immunogenic, whereas no live virus was found at the site of injection[58].

Ad vaccines

Ad vaccines have been used since the 1950s in military trainees. Adenoviruses among recruits were estimated to be the cause of about 70% of all respiratory illness and Ad was implicated in 90% of the pneumonia-related hospitalizations. Serotypes Ad4 and Ad7 were the first to be targeted for vaccination, due to their high prevalence among military personnel[44]. In the 1970s the first live oral Ad4 and Ad7 vaccines were tested and found to be safe and over 95% effective in preventing acute respiratory disease[507]. Studies with live adenovirus serotypes 4 and 7 vaccines in military recruits have demonstrated little horizontal transmission among military personnel, but substantial transmission among family members[42]. Shedding of oral Ad4 and Ad7 vaccine in stool samples was found from 7-21 days postvaccination[43]. Vaccines against Ad1, Ad2, Ad5[508] and Ad21[509] were next to be developed and although the effects were more modest in inducing neutralizing antibodies than natural infection, the overall immunogenicity, safety and shedding in stool samples were comparable to data from Ad4 and Ad7 studies.

Due to their high immunogenicity, adenoviruses are also very popular as vaccine vectors for induction of immunity against transgenic proteins of other viruses, such as from HIV, hepatitis B and influenza, but also against tumor-associated neoantigens present in certain cancers[510]. However, oral delivery of the Ad vaccines results in a relatively weak adaptive immune responses to the transgenes, whereas a robust immune response is observed against replication competent Ad vaccine vectors themselves[44].

Vaccinia-based vaccines

Poxvirus vector-based vaccines are being developed to protect against infectious diseases and treat cancer. As recombination between vaccine virus and naturally occuring orthopoxviruses *in vivo* might result in hybrid viruses with unpredictable characteristics. Recently, an *in vitro* study was performed assessing the effects of co-infection and possible recombination and demonstrated that indeed homologous recombination between poxvirus-vectored vaccine and naturally circulating poxviruses occurred, resulting in genetic instability of the transgene, accumulation of non-transgene expressing vectors and hybrid virus progeny[511]. Follow-up of US military personnel vaccinated with smallpox vaccine, showed that, despite provided printed information on prevention of transmission, including advice on hand washing, covering of the vaccination site and limited contact with infants, transmission of Vaccinia was 7.4 per 100,000 primary vaccinees, as confirmed by viral culture or PCR. This included predominantly secondary transmission and in two cases tertiary transmission to persons with close contact (within the same household, intimate contacts and sports partners)[52]. In a subsequent study, it was shown that all of the environmental swabs taken from of the recently vaccinated Vaccinia-negative persons bed linen, bath towels, shirt sleeves adjacent to the vaccination bandages and the vaccinees' hands were negative for live virus as determined by plaque infectivity assay, and only 0,78% of the injection-site bandages had measurable titers of Vaccinia[165]. These data underline that direct contact with live Vaccinia virus from the injection-site (bandages) is the main cause of secondary transmission.

In addition, Vaccinia virus (VACV) is commonly used in laboratories[79] and has been known to cause occasional infection of non-vaccinated laboratory personnel[167,512,513]. Most recombinant VACV strains are generated through insertional recombination in the viral TK locus[514,515]. Although TK is non-essential for viral replication, deletion results in a modest attenuation in virulence as shown in two mouse models[309,516]. Nevertheless, several human infections with TK-minus VACV following laboratory-related exposure have occurred[512,517-519], demonstrating the difference in virulence in animals and humans. Even more, some of the laboratory-acquired VACV infections have involved strains carrying foreign genes[512,517-519]. Exposure to VACV was in most cases the result of a needle-stick injury, an eye splash or while working with animals and resulted in fever, erythema and/or local swelling and needed in a few cases hospitalization[79]. These data considered, it is now recommended, at least in the USA, that in addition to appropriate laboratory measures, all laboratory personnel handling VACV be vaccinated, at least every 10 years[520] as the benefits of vaccination outweigh the risks of infection. In addition, the usage of Biosafety level 2 practices and facilities are recommended for manipulation of viruses or animals with VACV strains[79].

Non-gene therapy vaccines

Development of live attenuated vaccines to prevent severe wild type infections in the population may occasionally result in transmission of the lesser virulent vaccine virus strains from vaccinated children to unvaccinated contacts, thus contributing to herd immunity, but simultaneously also posing a possible risk of vaccine-derived disease in immunocompromised contacts.

Rotaviruses and orthoreoviruses are both members of the *Reoviridae* (Respiratory Enteric Orphan viruses).

The reoviruses differ from all other RNA viruses in that their genomes are double stranded. Rotavirus is a common cause of gastroenteritis in young children (<5 years of age), but since immunity to rotavirus is incomplete, outbreaks and recurrent infections during adult life may occur, especially in institutionalized elderly. Vaccine studies have shown that viral shedding and transmission to bystanders was more pronounced with the old tetravalent rhesus rotavirus vaccine in comparison to the newer human attenuated monovalent rotavirus vaccine, Rotarix (GlaxoSmithKline) and the pentavalent bovine-human reassortant vaccine, RotaTeq (Merck)[521]. However, Rotarix still has a much greater tendency to shed vaccine virus into the stools and is measured, depending on the dose, at 7 days after the first dose in 35-80% of the vaccine recipients, whereas only about 13% of recipients of RotaTeq were found to shed any component of the vaccine virus from day 4-6[522]. In two trials in infants conducted in Singapore[523] and the USA[524] transfer of Rotarix vaccine occurred to 3 and 2 placebo recipients, respectively. In contrast, shedding of live virus was not found in any of the healthy adults, who were administered a single dose of Rotarix[525].

The family of *Picornaviridae* consists of some of the smallest viruses, including Poliovirus, Coxsackie A and B virus, Echovirus and Enterovirus. Poliovirus is transmitted primarily by the fecal-oral route, but can also be excreted through respiratory droplets. When the virus invades the central nervous system, it causes paralysis due to destruction of motor neurons. The OPV vaccine strains are live attenuated and can replicate in the gastrointestinal tract, but not in the central nervous system. Ten recent outbreaks of poliomyelitis were shown to be caused by pathogenic circulating vaccine-derived polioviruses[526]. Most of these virulent viruses were found to be recombinants of mutated poliovaccine strains and other unidentified enteroviruses of species C. In addition some sequences in the 3' half of the recombinants was shown to bare homology to sequences of the co-circulating Coxsackie A17 strain. *In vitro* data demonstrated that recombination between the live attenuated polio vaccine and Coxsackie A17 resulted in viable and virulent hybrid progeny. These data emphasize that co-circulation of different viruses in the pediatric population can result in genetic recombination of viruses, despite differences in their pathogenicity and biological properties, including receptor usage, and lead to the generation of pathogenic recombinants[526]. Furthermore, a recent study compared the immunogenicity of monovalent type 1 oral poliovirus (mOPV1) vaccine and trivalent oral poliovirus (tOPV) in newborns and found that 36% of the children receiving mOPV1 and 42% of the newborns receiving tOPV shed vaccine strains in stool at any time[527]. Analysis of the vaccine strains showed intratypic differentiation with high numbers of antigenically divergent (AD) P1 isolates, including substitutions of amino acid 60 of the VP3 region and residue 99 of the VP1 region, particularly in the mOPV1 study group. Substitutions of residues in the capsid region as a result from high immunogenic pressure may play a role in reversion of attenuation and evolution of a neuro-virulent vaccine-derived polio virus (VDPV). Therefore, it was stressed that in areas with low vaccination coverage[528], vaccination with mOPV1 could potentially lead to transmission of AD P1 isolates and increase the risk for the development of VDPV[527].

Live attenuated influenza vaccine (LAIV1) is a trivalent nasal vaccine for active immunization and prevention of influenza, which consists of 3 cold-adapted, temperature-sensitive, attenuated virus strains. The influenza A and B master donor virus (MDV) strains used to develop this vaccine are genetically and phenotypically stable and undergo regular antigenic updating by replacing the 2 hemagglutinin and neuraminidase genes of the MDV strains with those of contemporary influenza strains[529]. Shedding and transmission of LAIV1 was tested in children aged 9-36 months, going to primary day-care. This setting was chosen to serve as a "worst-case" transmission scenario as it was previously shown that young children without prior immunity to influenza shed vaccine virus at higher titers and for longer duration than older children or adults, and because rates of transmission of wild-type influenza viruses and other infectious agents in the day-care environment are high[530]. It was found that 80% of the vaccinated children shed at least one virus strain for an average of 8 days during the 3-week post-vaccination period, which resulted in the confirmed transmission of vaccine strain to one placebo treated toddler, making the probability of vaccine strain transmission to a child after contact with a single vaccinated child 0.58%[531].

Transmission of vaccine strains from live attenuated Varicella vaccine (Oka strain) has been reported on few occasions. These included secondary transfer from a primary vaccinee to two immune competent patients in a chronic care facility for children[532], from a vaccinated mother to her two susceptible children[533], from a healthy 12-month old infant to his pregnant mother[534], and from a 5-year old boy in remission from ALL, but receiving maintenance anti-cancer chemotherapy, to his two siblings[535]. The latter is an excellent example of the risks of transmission after vaccination of immune compromised

patients. A study performed in children with leukemia, immunized with Varicella vaccine, demonstrated the importance of skin lesions in transmission to healthy susceptible siblings[536]. Out of 482 immunized children, 156 developed a rash, which resulted in secondary transmission to 17% of the exposed healthy susceptible siblings with mild disease and one case of tertiary transmission. The risk for transmission of the varicella vaccine to the exposed siblings was directly correlated to the number of skin lesions of the vaccinee[536]. Current recommendations from the Infectious Diseases Working Party of the EBMT for immunization of recipients of stem cell transplantation with Varicella vaccine therefore include either vaccination of seronegative patients before stem cell transplantation or at two years after SCT as well as vaccination of seronegative family members[537].

All together, these data suggest that 1) live attenuated vaccine shedding is associated with a low degree of immunity and a larger vaccine dose; 2) transmission is associated with intensive contact and most likely to occur in pre-immune children in daycare centers or within the same household, elderly and disabled people with suboptimal immunity housed in nursing homes and chronic care facilities, and among military trainees; 3) shedding and transfer is more likely to occur from the primary site of vaccination or vaccine-induced skin lesions; 4) recombination risk is associated with a low degree of immunity and occurrence of multiple simultaneous infections, and may result in viable and (more) virulent virus strains with different tropism.

APPENDIX D PROJECT EXECUTION

List of interviews with experts

- Dr. Annemieke Aartsma-Rus, Department of Human Genetics, LUMC, Leiden, The Netherlands: Immunogenicity of non-viral vectors
- Prof. Dr. Ben Berkhout, Center for Infection and Immunity Amsterdam, The Netherlands: HIV and use of lentiviral vectors
- Dr. Chiara Bonini, Experimental Hematology, HSR, Milan, Italy: Immune responses in bone marrow transplantation patients and immunotherapy
- Drs. Anna de Goede, Department of Virology, Erasmus MC, Rotterdam, The Netherlands: Shedding and transmission
- Prof. Dr. Rob Hoeben, Department of Molecular Cell Biology, LUMC: Stealth mechanisms and vectors
- Dr. Peter van der Ley, Laboratory of Vaccine Research, Netherlands Vaccination Institute, Bilthoven, The Netherlands: Risks associated with vaccinations
- Dr. Per Ljungman, Department of Medicine, Karolinska Institute, Sweden: Risk of infection after stem cell transplantation
- Dr. Marco Schilham, Department of Pediatrics, LUMC, Leiden, The Netherlands: Immunity and infections with Adenoviral vectors

Meetings with the supervisory committee and project progress

During the three meetings with the supervisory committee, the contents and progress of the report were discussed and adapted, where necessary.

- January 2009: Initiation project and discussion initial project plan by executers results in inclusion all relevant viral vectors, both replication deficient and replication competent, information on risks of immune modulation, shedding and recombination from non-gene therapy studies, such as vaccination and transplantation studies, as suggested by Prof. Dr. Rob Hoeben, and effects of currently used immune modulation in relevant preclinical gene therapy studies.
- May 2009: Data from clinical non-gene therapy are presented and discussion on project scope and focus
- September 2009: Presentation prefinal report

Attended meetings and publications

- ESGCT 2008, Brugge, Belgium
- Fatima S.F. Aerts Kaya, Leonie C.M. Kaptein, Gerard Wagemaker. Influence of immune modulation gene therapy on environmental risk assessment. Fifth annual CONSERT meeting, Leukerbad 2009. Poster presentation.
- DAVS 2009, Amsterdam, The Netherlands
- Fatima S.F. Aerts Kaya, Leonie C.M. Kaptein, Gerard Wagemaker. Influence of immune modulation gene therapy on environmental risk assessment. ASGT 2009, San Diego, USA. Mol Ther, May 2009; 17(s1), 381: abstract # 1000. Poster presentation.
- EBMT WP infectious complications 2009, Rome, Italy.
- Fatima Aerts-Kaya, Leonie Kaptein, Gerard Wagemaker. Lessons from non-gene therapy studies to predict the environmental risk in immune modulation gene therapy. ESGCT 2009. Hum Gen Ther 2009: 20(11), 1470. Abstract # 148. Poster presentation.
- Fatima S.F. Aerts Kaya, Leonie C.M. Kaptein, Gerard Wagemaker. Influence of immune modulation gene therapy on environmental risk assessment. Second annual PERSIST meeting, Leukerbad 2010. Oral presentation.
- Fatima S.F. Aerts Kaya, Leonie C.M. Kaptein, Gerard Wagemaker. Influence of immune modulation gene therapy on environmental risk assessment. NvGT 2010. Oral presentation.

Keyreferences

This section contains some highly relevant references, which were recently published during the preparation of this manuscript.

- Nayak and Herzog. Progress and prospects: Immune responses to viral vectors. Gene Therapy 2009: 1-10.
- Arruda, Favaro and Finn. Strategies to modulate immune responses: A new frontier for gene therapy. Molecular Therapy 2009: 17(9), 1492-1503
- Gene Therapy Immunology. Wiley-Blackwell 2009. Edited by Roland Herzog.

LIST OF ABBREVIATIONS

AAV	Adeno-associated virus
Ad	Adenovirus
ADA	Adenosine deaminase
APC	Antigen Presenting Cell
ATG	Anti-thymocyte globulin
Bu	Busulfan
CAR	Coxsackie-adenovirus receptor
CMV	Cytomegalovirus
CR	Conditionally replicating
CsA	Cyclosporin A
CTL	Cytotoxic T cell
CTLA4	Cytotoxic T cell antigen-4
CY	Cyclophosphamide
DC	Dendritic Cell
EBV	Eppstein Barr Virus
ERA	Environmental Risk Assessment
FDA	Food and Drug Administration
GALV	Gibbon Ape Leukemia Virus
GFP	Green Fluorescent Protein
HIV	Human Immune deficiency Virus
Hd-Ad	Helper dependent Adenovirus
HSV	Herpes Simplex Virus
IA	Intra arterial
IFN	Interferon
IL	Interleukin
IP	Intraperitoneal
IRES	Internal ribosomal entry site
IT	Intratumoral
IV	Intravenous
lacZ	b-galactosidase
LV	Lentivirus
M Φ	Macrophage
MHC	Major Histocompatibility Complex
MLV	Murine leukemia Virus
MMF	Mycophenolate Mofetil
MMP	Matrix metalloproteinases
MTX	Methotrexate
MV	Measles Virus
NAb	Neutralizing antibody
NDV	Newcastle Disease Virus
NK	Natural Killer cell
OV	Oncolytic Virus
PAMP	Pathogen activated molecular patterns
PCR	Polymerase Chain Reaction
pfu	plaque forming unit
Polio	Poliovirus
PPR	Pattern Recognition Receptor
RC	Replication Competent
Reo	Reovirus
RV	Retrovirus
SCID	Severe Combined Immune Deficiency
Th	T-helper cell
TK	Thymidine Kinase
TLR	Toll-like receptor
TNF	Tumor Necrosis Factor
VACV	Vaccinia Virus
vp	viral particles
VSV	Vesicular Stomatitis Virus
WNV	West Nile Virus

Table I. Immune suppression used in clinical trials and animal studies with *ex vivo* gene transfer

Human trial	Immune suppression	Delivery	Animal model	Immune suppression	Construct
ADA-SCID	2 mg/kg/d Busulfan at day -3 and -2 before GT	Autologous CD34+ BM-MNC transduced with RV-GIADA*l*[538]	Adult (5wk) ADA-deficient mouse (FVB) treated with PEG-ADA	600 cGy radiation	Congenic BM transduced with LV-ADA[224]
			Neonatal (5d) ADA-deficient mouse (FVB)	300 cGy radiation	Congenic BM transduced with LV-ADA[539]
X-CGD	None	Autologous CD34+ PB-MNC transduced with RV-gp91phox or p47phox [232]	X-CGD mouse (C57)	4 µg/d 2dd G-CSF at day -4 to -1, 4 µg G-CSF at day 0 + 300 cGy TBI	Congenic BM transduced with RV-gp91phox[233]
			NOD-SCID mouse	300 cGy TBI at day -2 + 1 mg TM-β1 antibody (anti-CD122) at day 0	Human X-CGD PBSC transduced with LV-gp91phox[225]
			Hemophilia A mouse	100 mg/kg/d CY, 17.5 mg/kg BU on day -3 and -2 ip, 30 mg/kg FLU on day -3 to -1 ip or 300 cGy TBI plus 0.5 mg CTLA4Ig and anti-CD40L ip at days 0 and +2 or ATS ip at day -1 and 0.	Sca1+ BM cells transduced with MSCV-BDDpfVIII[404]

Table II. A Immune modulation used in animal studies using *in vivo* delivery of replication deficient vector

Animal model	Immune modulation	Dose/Construct/ROA	Effect immune modulation	Vector persistence and biodistribution	Transgene expression	Ref
8-10 wk old C57Bl/6	0.1-10 mg/kg Dexamethasone at -15 hrs and -2 hrs ip	$0.75x10^{11}$ vp E1-deleted Ad5-L*acZ* iv	Dose dependent suppression of IL-6, MIP1β, MCP1, IL-12, KC, LIX, RANTES, reduced neutrophil infiltration and innate & adaptive immune response	Similar transduction efficiency Increased copy number in liver (RT-PCR) Decreased levels of Ad NAb Reduced level of Ad clearance	Similar transgene expression levels Reduced levels of anti-transgene NAb	197
2-3 month old C57Bl/6 and Balb/c mice	0-4 mg Methylprednisolone at -6 hrs and 0 hrs	10^{11} vp E1/E3/E4-deleted (PEGylated) $AdAT_4$-ApoA-I iv	Suppressed levels of IL-6, MCP1, MIP-2, MIB1β, IP-10, LIX, KC, reduced neutrophil infiltration, PEGylation prevented thrombocytopenia	Increased copy number in liver	Similar transgene expression levels	55
C57Bl/6 or NOD-SCID	100 μg HAM-CD3 ip, d-1, d+0 and d+1	10^{11} vp E1-deleted Ad5Amy-*LacZ* iv	Cellular immune response averted	ND	ND	398
8-12 wk old Lewis rats	2 mg/kg iv 2x/wk MAR-ICOS JTT.1 mAb from d+0 to d+120	$1-5x10^{10}$ vp E1-deleted AdCD40Ig intracardiac injection	Suppressed levels of IL-2 and IFNγ Decrease in IL-10	ND	ND	436,540
8-12 wk old Lewis rats	4 mg/kg/d ip MAR-CD28 JJ319 mAb from d+0 to d+7	$5x10^{10}$ ip E1-deleted AdCD40Ig in the portal vein	Reduced transcripts for IL-6, perforin, CTLA4, HO-1, TGFβ and IDO	ND	ND	426
Adult male Sprague-Dawley rats	300 μg/d murine CTLA4Ig ip from d+0 to d+2	50-100 μl of $8x10^{10}$ pfu/mL E1-deleted Ad-WTp53 in carotid artery	ND	ND	CTLA4Ig increased the effect of p53, no difference between 1, 2 or 3 doses of CTLA4Ig	415
7-8 wks old female C57Bl/6	100 μg/d human CTLA4Ig at d+0 to d+4	$5x10^{8}$ pfu in trachea or $1x10^{9}$ pfu E1-del Ad.H5.010CMV*lacZ* iv	CTLA4Ig blocked NAb, decreased anti-vector CD4 and CD8 T-cells	ND	CTLA4Ig moderately prolonged transgene expression: liver > lung	416
30-50 days old *mdx* mice	5 mg/kg/d sc FK506 from d-1 to end experiment and human 200 μg CTLA4Ig ip at d+0, d+2 and d+10	20 μl of $7x10^{11}$ vp/ml of E1/E3-deleted Ad5-Dys im	Reduced inflammation, reduced anti-Ad antibodies, reduced anti-transgene antibodies	increased transduction efficiency	Prolonged transgene expression	417
2 month old Sprague-Dawley rats	40 μg/kg anti-murine CD4 and CD8 mAb im or 40 mg/kg CsA im	$1.5x10^{7}$ pfu Ad-hBMP9 im	Anti-CD4 and CD8 transiently reduced WBC levels and decreased anti-Ad Nab	ND	Anti-CD4 and CD8, but not CsA increased bone formation	71

Table II. A Immune modulation used in animal studies using *in vivo* delivery of replication deficient vectors (cont).

Animal model	Immune modulation	Dose/Construct/ROA	Effect immune modulation	Vector persistence	Transgene expression	Ref
Adult male Lewis (recipients), DA rats (donors)	1 mg/kg every other day for two weeks MAR-ICOS mAb (JTT.1)	$1x10^9$ pfu Ad-CTLA4Ig iv	Induction of stable tolerance, Decreased heart rejection, no effect on memory T-cells	ND	ND	418
4-7 year old *macaca mulatta*	10 mg/kg anti-human CD40 with or w/o 10 mg/kg anti-human CD86 at day -3, and 5 mg/kg mAb at days 0 and +3 iv	$5x10^{10}$ iu/kg E1 and E3-deleted Ad5-mγIFN or Ad-hsCD4 at day 0 and day +32 iv	Delayed or blocked humoral reponses against Ad, decreased CD8+ CTL infiltration in liver	ND	Prolonged transgene expression	434
C57Bl/6 mice	10.000 IU anti-mouse IFN-α and IFN-β ip at -6 hrs and day +5	$2x10^9$ pfu E1 and E3-deleted Ad-LacZ iv	Diminished virus-specific T-cell response Reduction of inflammation in the liver	Increase in viral DNA copies in the liver	More stable transgene expression	189
6-8 wks MPS VII C57Bl/6J mice	500 μg anti-CD40L (MR-1) ip at d-1, 0, +1, 2, 4, 6, 9, 12	$2x10^9$ pfu E1-deleted Ad5. βgluc iv	Delayed anti-Ad IgG	ND	Delayed anti-β-gluc IgG and prolonged β-gluc expression	435
6-10 wk mice	50 mg/kg CY and 10 mg/kg CsA ip 3x every two weeks for 8 weeks	$1x10^9$ pfu AdMCdF9 or AdMCLacZ in 100 μL PBS im	CsA alone had no effect on NAb	ND	CsA alone has no effect; CY with or w/o CsA prolonged transgene expression	541
fIX deficient dogs	19.5 mg/kg/d CsA po	$1\text{-}2.2x10^{11}$ pfu/kg Ad.RSV-cFIX into cephalic vein	CsA had no effect on NAb	ND	CsA prolonged transgene expression	542
Female C57Bl/6J	70 mg/kg CY iv day 0 and 4	$5x10^8$ pfu E1-deleted Ad5.010CMVlacZ in trachea or $1x10^9$ pfu iv	CY inhibits activation of anti-Ad CD4+ and CD8+ T-cells, reduced inflammation, inhibits upregulation of MHC class I	ND	CY prolonged transgene expression liver > lung CY prevented NAb in lung > liver	543
C57Bl/6J	2 μg IL-12 ip or in trachea at day 0 and +1; 1 ug IFNγ in trachea or 2 μg ip dat day 0 and +1; anti-CD4 mAb (GK1.5) at day -3, 0 and +3	$1x10^9$ pfu E1-deleted Ad5.010CBALP at d0 and at day +28 Ad5.010CMVlacZ via trachea	IL-12 blocked anti-Ad IgA, but not IgG; IFNγ decreased anti-Ad IgA and NAb; anti-CD4 mAb reduces pulmonary anti-Ad IgA	ND	Anti-CD4 mAb prolonged high level transgene expression	544

Table II. A Immune modulation used in animal studies using *in vivo* delivery of replication deficient vectors (cont).

Animal model	Immune modulation	Dose/Construct/ROA	Effect immune modulation	Vector persistence	Transgene expression	Ref
2.5-6.6 yrs old *macaca mulatta*	250 mg/m^2 rituximab, 2 doses 3 wks apart, 30-100 mg/kg/d CsA wk+23-27	10^{12} vp/kg scAAV2/8-LP1-hFIXco in mesenteric vein	ND	ND	Disappearance of anti-transgene NAb	396
Macaca mulatta	25 mg/kg MMF po, 2x/d, d-7 to d+70 plus 2-4 mg/kg sirolimus po up to d+70 with or w/o 2 mg/kg daclizumab at d-1 and 1 mg/kg at d+15	8x10^{12} vg/kg AAV2.hFIX in the hepatic artery	MMF+sirolimus suppressed anti-AAV2-antibody MMF+sirolimus+daclizumab increased anti-AAV2-antibody and reduced Tregs	No effect on transduction efficiency and vector copy number with either regimen, no effect on biodistribution of the vector	MMF+sirolimus: no effect MMF+sirolimus+daclizumab: Loss of expression	407
Male *macaca mulatta*	0.25-1.0 mg/kg FK506 and 25 mg/kg MMF po twice per day from d-3 to d+45	2x10^{13} vg/kg AAV8.hFIX in the hepatic artery	FK506 and MMF prevented development of NAb	Increased vector copy number in liver, some in spleen and rare in testes	Induction of tolerance to transgene (but not the to vector)	54
1.5-3 year old *cxmd* dogs	5 mg/kg 2xeucop po from day -2 to day +12, 7.5 mg/kg 2x/d MMF sc from day -2 or day 0-28, 1 mg/kg ATG from day -2 to day +2	1x10^{11} vg AAV6-CMV-h-μ-dys im	CsA + MMF decreased cellular infiltrates, but not affect T cell responses CsA + MMF + ATG decreased PBL	ND	CsA + MMF increased transgene expression CsA + MMF + ATG allows long-term transgene expression	405
7-12 months beagle dogs	10-30 mg/kg/d CY d-1 to d60 or d90 and 15 mg/kg/d MMF d0 to d60	2-3x10^{13} vg AAV6-ACAG-hAAT intratracheal spray	NAb to AAV6 capsid, but not to hAAT	ND	CY/MMF: prolonged expression of hAAT in lung and serum	545
6 wk old *mdx* C57Bl6 mice	6 mg/kg/d MR1 (anti-CD40 mAb) and 5 mg/kg/d CTLA4Ig ip at d+0, d+2 and d+4	10^{11} vg AAV1-U7 im	Inhibition of anti-AAV1-IgG and NAb up to 6 months	Blockade needs te be readministered for reinjection, increased transduction efficiency	Prolonged transgene expression	386
Hemophilia B C57Bl/6/129 mice	20-50 mg/kg CY ip at day 0 and biweekly up to 6 wks or 125 μg FK506 sc at day 0, every other day for 1 month or 50 mg/kg CsA ip, every two weeks for 1 month, 0.1 mg anti-CD40L and murine CTLA4Ig ip at days -3, 0, +3, +6, +9	1.0x10^{11} vg AAV2-CMV-F9 im	Anti-CD40L: no effect on Nab formation FK506 prevented formation of NAb for the duration of treatment and resulted in infections (!) CsA: death as a result of renal toxicity CY: prevented or decreased formation of NAb		Anti-CD40L or CTLA4Ig: transient partial correction of aPTT CsA: no improvement aPTT CY: long-term systemic expression of F9, partial correction of aPTT	546

Table II. A Immune modulation used in animal studies using *in vivo* delivery of replication deficient vectors (cont).

Animal model	Immune modulation	Dose/Construct/ROA	Effect immune modulation	Vector persistence	Transgene expression	Ref
2-8 yrs old male LPL -/- cats	100 or 200 mg/m^2 CY po, weekly from day -3 for 8 wks	1x10^{11}, 5x10^{11} or 1x10^{12} gc/kg AAV1-CMV-hLPL^{S477X} im at 10 injection sites	CY: prevented formation of NAb to LPL at low vector dose, but not at intermediate and high dose	ND	Local LPL expression in injected muscles, correction of triglyceride levels	547
F.IX missense mutation Hemophilia B dogs	5-10 mg/kg CY iv at day 0 and weekly from wk +2 to wk +6 and 100 mg Na-2-mercapto-ethane-sulphonate (Mesna)	5.6x10^{12} vg/kg AAV2-CMV-cfIX	CY: limited anti-cfIX antibody formation, but continued anti-AAV-2 NAb formation	ND	No effect on transgene expression, but more rapid decrease in clotting time	458
F.IX nonsense mutation Hemophilia B dogs	10 mg/kg CY at day 0 and 60 mg biweekly up to wk 6 and 100 mg Mesna	1x10^{12} vg/kg AAV2-CMV-cfIX	CY: blocked anti-fIX antibody formation, but continued anti-AAV-2 NAb formation	ND	Sustained transgene expression >8 months	236
F.IX missense mutation Hemophilia B dogs	200-250 mg/m2 CY iv at at d-1 or d+15 and then weekly for 6 wks	1x10^{12} or 2.4x10^{11} vg/kg AAV1-CMV-cF.IX im at multiple sites	Start d+15 CY: no inhibition of NAb against F.IX Start d-1 CY: prevention NAb formation	ND	d+15 CY: no effect on F.IX expression d-1 CY: transiently increased F.IX transgene expression	234
5-7 wks old male Sprague Dawley rats	0.02-1 mg/kg human CTLA4Ig every other day for 8 d and 3 mg/kg/d LF15-0195 ip from d+0 to +20	1.5x10^8 vp RV-LacZ iv	ND	Similar transduction efficiency at day 7	Dose dependent reduction of anti-β-gal NAb	413
6 wk MPS I C57Bl/6	25 mg/kg human CTLA4Ig ip at -6 hrs, at d+4, d+7, d+11 or 12.5 mg/kg ip α- CD40L antibody at d+1 and 3 or 12.5 mg/kg ip α- CD4 antibody at d+1 and 2	5x10^9/kg TDU RV-hAAT-cIDUA-WPRE iv	Transient CTLA4IG: transient immune suppression CTLA4Ig+anti-CD40L: decreased liver infiltration with lymphocytes	ND	CTLA4Ig+anti-CD40L: prevents anti-IDUA antibodies CTLA4Ig+anti-CD4: prevents anti-IDUA antibodies	414
Newborn MPS I cats	25 mg/kg human CTLA4Ig iv 2x/wk from d+0 to 15	1-10x10^9 TU/kg RV-hAAT-cIDUA-WPRE iv	CTLA4Ig effectively blocked the anti-IDUA CTL response	Increased RV DNA copy number in the liver	Induction of tolerance to canine IDUA	548

Table II. B Immune modulation used in animal studies using *in vivo* delivery of replication competent vectors

Animal model	Immune modulation	Dose/Construct/ROA	Effect immune modulation	Vector persistence	Effect on tumor	Ref
6 wk old A/J female mice	5 mg/kg/d Dexamethasone ip from day 0 to day +6 or from day -2 to day +14	$1x10^7$ pfu $\gamma_1$34.5-deleted, HSV-1 with *lacZ* gene inserted in the ICP6 gene (G207) at day 0 and day +3	Decreased levels of anti-HSv-NAb Decreased tumor-specific CTL response(!)	No effect on intra-tumoral viral replication		[549]
Adult nude (*rnu/rnu*) rats or Fisher 344 rats	60 U/kg Cobra Venom Factor (CVF) ip, day -1 and 20 U/kg at day 0 and 100 mg/kg CY ip at day 2	$2x10^9$ pfu HSV-1 with LacZ gene inserted in UL39 (hrR3) ia	ND	Vector DNA by PCR present in many organs, but no evidence of viral replication or viral gene expression	CVF facilitated transduction of brain tumors, CY enhanced the effects of CVF	[68]
4-6 wks old female C57Bl6 mice	300 mg/kg CY ip at day -2	$1x10^7$ pfu GFP inserted in pharmaco-kinetic domain of ICP10 gene of HSV-2 (FusON-H2), intratumoral	Decreased monocytic cell infiltration, no effect on lymphocytic cell infiltration Enhanced anti-tumor CTL activity	CY increased and prolonged intratumoral virus replication at day +2 and day +4 CY increased anti-tumor effect of FusON-H2	CY increased antitumor effect of FusON-H2	[550]
4-5 wk old Syrian golden hamsters	First dose 140 mg/kg CY, then 100 mg/kg CY 2x/wk	Pre-immunization with $1x10^{10}$ Ad5 twice, then $1x10^{10}$ E3-deleted, E3-11.6K (ADP) over-expressed Ad5 (INGN007) intratumoral, for 6 days	CY induced general leukopenia CY decreased NAb present in serum and in the tumor CY prevented formation of NAb and reduced pre-existing levels of NAb	CY elevated and prolonged presence of intratumoral infectious virus levels CY increased and prolonged presence of viral DNA in blood, liver and lung		[169,551]
6-8 wks old C57Bl6/J	150 mg/kg/d CY once or 3 days	$5x10^5$ to $5x10^8$ pfu Reovirus type 3 (Dearing) iv	3d CY resulted in high (cardio) toxicity if given at the same day as Reovirus, but less when given one day separated	1d CY did not increase virus levels in tumor, but virus was found in heart tissue, 3d CY increased levels of intra-tumoral virus and other organs	3d CY resulted in tumor regression	[14]
6-8 wks old C57Bl6/J with HPV16 transformed cells	200 mg/kg CY ip	$2x10^7$ pfu Reovirus type 3 (Dearing) intratumoral	ND	ND	CY suppressed growth of tumors better than Reo CY increased effects when given 7 days after Reo	[552]
Fisher 344 rats	5 mg/kg/d Rapamycin, 5d or 60 mg/kg Cy once	$1x10^9$ pfu TK-del, VGF-del, vvDD-EGFP (Vaccinia WR strain) ip	Both CY and rapamycin prolonged survival	Both CY and rapamycin increased intratumoral viral replication	Both CY and rapamycin increased intratumoral EGFP expression	[40]

Table II. C Structural modifications used in animal studies using *in vivo* delivery of replication competent vectors

Animal model	Modifications	Dose/Construct/ROA	Effect modification on immune system	Vector persistence and biodistribution	Effect on tumor	Ref
6-8 wk old C57Bl6J mice with melanoma	1) Fusogenic F3aa by modified F cleavage site 2) influenza NS1 protein works as IFN antagonist, anti-apoptotic function,	$5x10^6$ NDV(B1), NDV(F3aa) or NDV(F3aa)-NS1 intratumoral injection every other day for 4-6 days	NDV(F3aa)-NS1 suppressed innate immune response (delayed induction pro-inflammatory cytokines) and increased tumor-specific CTL response	ND	Fusogenic and cytolytic effects NDV/F3aa)-NS1 > NDV(F3aa) > NDV(B1) NDV(F3aa)-NS1 suppressed tumor growth and promoted survival	347
Mice	1) Hydroxypropyl-methacrylamide-coated 2) retargeted with cetuximab to bind EGFR	$5x10^{10}$ VP Ad5WT, p-Ad5WT, or Cx-p-Ad5WT, 3 doses every 72 hrs ip	Decreased peritoneal adhesions due to ip Ad injection	ND	Decreased tumor volume and increased survival	553

Table II. D Immuno deficient animal studies using *in vivo* delivery of replication defective vectors

Animal model	Dose/Construct/ROA	Tumor/Disease model	Vector persistence and biodistribution	Shedding/Transmission	Transgene expression/effect on tumor	Ref
CD4 knockout C57Bl/6 mice	AAV1-CMV-fIX or AAV2-CMV-fIX at $2x10^{11}$, $1.2x10^{12}$, $4x10^{12}$ vg/kg im at 4 sites	Hemophilia B	Southern blot: gene copy number of AAV1> AAV2 in muscle	ND	increased vector dose, increased F.IX transgene expression, AAV1 > AAV2	234
Female 5 wk old Balb/c nu/nu mice	$2x10^8$ pfu E1, E3 del AxCAUT it	10^7 HuCCT1 human cholangiocarcinoma cell line in the right site of the abdomen or ip			Decreased tumor volume	554
8-10 wks old C57Bl/6 Rag2-/- mice	AAV6-ACAG-hAAT at $1\text{-}2x10^{11}$ vg by nasal aspiration	Alpha1-antitrypsin deficiency	ND	ND	Stable hAAT expression in lung and plasma for > 1 yr in Rag2-/- mice, but < 6 months in normal mice	545

Table II . E Immuno deficient animal studies using *in vivo* delivery of replication competent viral vectors

Animal model	Dose/Construct/ROA	Tumor model	Vector persistence and biodistribution	Shedding/ Transmission	Transgene expression/effect on tumor	Ref
Female 4 wk old Beige/ SCID mice	$1x10^8$ pfu Ad-CMV-fl or AdMUC1-TSTA-fl iv	$5x10^5$ KPL-1 human breast cancer cell line into the second mammary fat pad	Local transgene expression in draining lymphnodes	ND	Luciferase expression after AdCMV-fl in liver No luciferase expression after AdMUC1-TSTA-fl in liver, but persistent intratumoral transgene expression	555
Female 5 wk old Balb/c nu/nu mice	$2x10^8$ pfu AxE1CAUT it	10^7 HuCCT1 human cholangiocarcinoma cell line in the right site of the abdomen or ip	ND	ND	Decreased local tumor volume and peritoneal dissemination and prolonged survival	554
Athymic rats	$1x10^7$ pfu NV1066 (ICP-0, ICP-4 and $y_1$34.5 del HSV-1-CMV-eGFP) it	$1x10^7$ CWR prostate cancer cell line sc in flank	Only tested in tumor tissue, increased vector pfu in non-orchiedectomized rats	ND	ND	556
Female 6-8 wk old CD-1 nude mice	$5x10^6$ pfu vMyx-gfp it	$5x10^5$ U87 or U251 glioma cells ic	Detection of myxoma virus protein by IHC in residual tumor tissue only	ND	Intratumoral GFP expression, decreased tumor volume and prolonged survival	557
6-8 wk old athymic Balb/c nu/nu mice	$1x10^7$ pfu Δ24-E1A/del E3 Ad5 it once, $5x10^8$ pfu 5 times or $1x10^9$ pfu 4 times	10^7 D-54 MG human glioma cells (lack p16) sc	ND	ND	>66-86% tumor reduction	558
4-6 wk old female athymic nu/nu mice	10^8 pfu it,5 days *dl*922-947 (E1A mutant), *dl*1520 (ONYX-015), *dl*309 or wt Ad	$2x10^6$ C33A or HLaC human carcinoma cells sc	Viral replication in tumor	ND	Tumor regression *dl*922-947 > ONYX-015 > *dl*309 or wt Ad	559
4-6 wk old female athymic nu/nu mice	$2x10^8$ pfu iv, 5 days *dl*922-947 (E1A mutant), *dl*1520 (ONYX-015)	$2x10^6$ SW620 colon cancer cells sc or 10^6 metastatic MDA-MB-231 breast cancer cells into mammary fat pad	Viral replication in tumor tissue and lymphnode metastases	ND	Decrease in lung and lymphnode metastases *dl*922-947 > wt Ad > ONYX-015	559
Athymic mice	$1.5x10^8$ pfu Δ24-E1A Ad, RA55 (E1B-55 del) Ad it 10^9 pfu, 4x Δ24-E1A Ad, RA55 (E1B-55 del) Ad it	$5x10^5$ human glioma U-87 MG or U-251 MG ic 10^7 D-54 MG sc	Intratumoral replication Δ24-E1A Ad > RA55	ND	Survival Δ24-E1A Ad > RA55 Inhibition tumor growth Δ24-E1A Ad > RA55	560
Female athymic CD1 nu/nu mice	$1x10^7$ pfu HSV-1 (G207) it or $2x10^7$ pfu iv w or w/o 0.5 mg/kg vincristine iv	$1x10^7$ human rhabdo-myosarcoma cell lines KFR or KF-RMS-1 sc	Intratumoral replication	ND	Inhibition of tumor growth it > iv	561
Female 6 wk old nod/ scid, Balb/c nu/nu mice, CB17 scid /scid, CB17 scid/ beige mice	25 µl of $5x10^7$ pfu/mL HSV-1-RFP	$1x10^6$ murine oral cancer cell line AT-84 cells in floor of the mouth	Small and brief increase in virus present on d+5 in the nu/nu, scid/scid, and scid/ beige mice, but not > input quantity	ND	ND	66

Table III. Systemic immune suppression

Immune suppressants	Example	Innate	Adaptive	Viral infection
Glucocorticoids	Methylprednisolon Dexamethasone	inflammatory CK ↓	Recruitment and activation T-cells↓	HBV, HCV ↑
Cytostatic drugs				
Alkylating agents	CY, Bu	↓	Inhibition of B-cells, CD4+/CD8+ T-cells	VZV and CMV ↑[387]
Anti-metabolites	MTX, AZT, Flu		General bone marrow depression	EBV ↑[385]
Anti-mitotic agents	VC, Paclitaxel			
Cytotoxic antibiotics	Daunorubicin, Doxorubicin			AAV↑
Antibodies				
Polyclonal Ab	ATG	NK, DC, mono ↓[399]	Long lasting T and B-cell depletion	EBV↑, CMV ↑, BK ↑[400], HIV ↑
	IvIg		T and B-cell effector function ↓	
Monoclonal Ab	anti-CD25 (Daclizumab, IL-2Rα)		Minimal depletion of T-cells, Tregs ↓	ST: CMV ↓, LT: CMV ↑[406]
	anti-CD3 (TCR)		ST T-cell depletion	CMV ↓, EBV ↓, HHV6 ↓[397]
	Alemtuzumab (anti-CD52)	NK, mono ↓	LT T-cell and B-cell depletion	AdV ↑[390], CMV ↑, BK ↑[388,389]
	Rituximab (anti-CD20)		B-cell depletion	*[412]
Blockade of co-stimulation	CTLA4Ig, LEA29Y		Blocks CD28-B7 interactions	*[412]
	anti-ICOS		Blocks ICOS/ICOS-L interactions	no data available
	IDO		T-cell apoptosis, T-cell proliferation ↓	no data available
	anti-CD28		Blocks CD28-B7 interactions	no data available
	CD40Ig		Blocks CD40-CD40L interactions	no data available
Calcineurin inhibitors	Cyclosporine (CsA)		T-cell proliferation and differentiation↓	CMV ↑[381] HCV↓
	Tacrolimus (FK506)		T-cell activation ↓	
mTOR (mammalian target-of-rapamycin) inhibitors	Sirolimus Everolimus		T-cell effector function ↓, Tregs ↑	BK ↑, CMV ↓
Interferons	IFNα-2a, 2b	Activation of mono ↓	Th1 CK ↓	
TNFα-blocker	Infliximab, etanercept and adalimumab	IL-1 ↓, IL-6 ↓	Lymphocyte activation ↓	*[562]
IL-1R-antagonist	Anakinra			§[412]
Mycophenolate mofetil	MMF		Specific and potent inhibition of T and B cell proliferation	BK ↑, CMV ↑, EBV ↓, HBV ↓, HIV ↓[490], HCV↓
Proteasome inhibitor	Bortezomib	Neutropenia		VZV ↑[446]

ST: short-term; LT: long-term; * not associated with an increased risk of viral or bacterial infections; § associated with increased bacterial infections, but not with increased risk of viral infections

Table IV. Characteristics of the wild-type virus used as parental strains for vectors

	HAdV	AAV	HSV-1	RV	LV	Vaccinia	Measles	Reo virus	NDV
Genome	ds DNA	ss DNA	ds DNA	Retro	Retro	ds DNA	(-) RNA	ds RNA	(-) RNA
Life cycle	24 hrs		12 hrs			8 hrs		18 hrs	18 hrs
Incubation period	5-8 days		3-7 days			7-9 days	8-12 days	2-4 days	2-15 days
Infectivity of non-replicating cells	Yes	Yes	Yes	No	Yes	Yes		No	No
Integration	No	Yes or episomal	No	Yes		No			No
Pathogenicity	Some serotypes	No	Yes	Yes		Attenuated disease	Yes	No	No
Immunogenicity	High	Low	Moderate	Low	Low	High	High	Low	High
Innate immune response	TLR9, complement	TLR9, no PAMPS, complement and activation of MΦ	TLR2, 3 and 9		TLR3/7 Trans-duction of DC	TLR2, complement and activation APCs	TLR2	RIG-1/Mda-5	RIG-1/ Mda-5
Cytokines involved	IFNα/β, IL-1, -6, -8, -12 and TNFα	TNF-α, RANTES, IP-10, MIP-1β, MCP-1 and MIP-2	IFNα/β, γ, IL-1, -2, -4, -5, -6, -10, -12, -23 and TNFα		IFNα/β	IFNα/β	IFNγ, IL-4	IFN-γ, IL-10	IFNα, TNFα
Viral clearance	CTL mediated	CTL and NAb mediated	CTL mediated	Antibody mediated		primary inf: CTL second inf: NAb	CTL	NAb, CD4+ and CD8+ T-cells	NAb and CTL response
Evasiveness	Latency	Integration	High, latency	Variability in antigenic structure			Suppression of IFN	Latency	
Transmissibility, shedding	Respiratory (aerosol), stool	Respiratory	Saliva	Body fluids, blood products		Respiratory, direct contact	Respiratory	Respiratory	Respiratory, fecal/oral
Cell lysis/fusion	Lysis	Depends on helper virus	Fusion	Fusion (budding)		Lysis	Fusion	Lysis	Lysis/fusion depends on strain
Tropism	Broad, depends on serotype	Broad	Restricted, CNS, PNS	Restricted		Broad	Broad	Respiratory tract, GIT, CNS	
Host	Human	Human	Human	Animal	Human	Human	Human	Human	Avian
Physical stability	Stable at RT for up to 3 wks, resistance to chemical and physical agents	Heat and pH stability, solvent resistance	Exceptional structural and mechanical stability				Labile, sensitive to acid, proteol enzymes, strong light and drying Stable for few hrs as droplet		Stable for 3 wks in water Sensitive to radiation, oxidation, acid, alkalis, phenols and direct sunlight
Recombination	Yes		Yes	Yes		Yes	No		No
Antiviral agent	No	No	Acyclovir, Ganciclovir	No		Cidofovir, Vaccina Ig	Mycophenolic acid		No
References	176,190					295	563	335	347

Table V. Categories of transgenes

Category	Example	Environmental risk increased
Structural proteins	Actin, myosin	No
Enzymatic proteins	Proteases, phosphatases	No
Metabolic enzymes		No
Cell growth and house keeping proteins		Possible
Cell cycle and division proteins		No
Proteins involved in DNA replication		Yes
Membrane proteins	Ion channels, G-coupled receptors, transporters	Possible
Proteins enhancing cytotoxic or lytic activity	Fusogenic membrane proteins	Yes
Tracking	eGFP, luciferase	No
Selection	MGMT	No
Antibiotic resistance	Neomycin	Possible
Prodrug activating	f-FC/CD	No
Toxins	Botulin	Yes
Regulatory genes, transcription factors		Yes
Growth factors, cytokines, chemokines		Yes
Immune modulatory molecules	CTLA4Ig, B7.1	Possible
Oncogenes		Yes

TableVI. Properties of viral vectors

Vectors	Vector modifications	Host range	Vector yield (TU/mL)	Inflammatory potential	Tropism	Entry pathway	Vector genome forms	Transgene expression	genotoxicity	shedding
AdV -Ad2, 5, 7	E1 and E3/E4-deleted	Broad, depending on serotype	High 10^{12} PFU/mL	High	Broad, depending on serotype	Receptor-mediated endocytosis	episomal	Short-term (wks)	low	moderate
RV - MMLV	*gag, pol, env*-deleted	Ecotropic Amphotropic VSV-G Ψ	Moderate 10^{10}	Low	Dividing cells Broad	Receptor–binding, membrane fusion	integrated	Long-term (yrs)	Insertional mutagenesis	low
LV - HIV, SIV, EIAV, FIV	*gag, pol, env, nef, vpr*-deleted	Ecotropic Amphotropic VSV-G Ψ	Low-Moderate	Low, depends on envelope proteins	Depends on envelope proteins		integrated	Long-term (yrs)	Insertional mutagenesis	low
Herpes - HSV-1	non-lytic	Broad	Moderate – High 10^{10}-10^{12}	High	Neurons	Receptor-binding, membrane fusion	episomal	Transient	low	high
AAV -AAV1, 6-9 -AAV8, 9 -AAV1, 4, 5 -AAV2 -AAV8 -AAV9	rep-, cap-	Broad	High 10^{12}	Low	Broad Muscle Liver CNS Kidney Pancreas Lung	Receptor-mediated endocytosis, endosomal escape	episomal (90%), integrated (10%)	Medium to long-term (yrs)	Low, but conceivable insertional mutagenesis upon integration	moderate
Pox - Vaccinia - Canary		Broad	10^{8} PFU/mL	High			episomal	Transient	low	high
Measles										
Baculovirus		Mammalian	High titer			Broad				
Reovirus type 3			10^{8} – 10^{9} PFU/mL		Broad, tumor cells	Receptor-mediated endocytosis	episomal		none	

Adapted from viral vectors, from virology to transgene expression. TU : transducing units *http://www.stanford.edu/dept/EHS/prod/researchlab/bio/docs/viral_vectors.pdf*

Table VII. Properties of viruses that determine transmissibility

Property	Feature
Survivabililty outside host	Resistance to ambient temperatures, drying, UV light, pH, physical or chemical agents
Existence of an alternative host	Mosquito, pets
Portal of entry	Skin, mucous membranes (respiratory tract, gastrointestinal tract, conjunctiva, genital tract)
Evasiveness	Rapid multiplication before induction immune response, high variability in antigenic structure
Pathogenesis	Incubation period
Route of excretion	Respiratory, conjunctival, skin/mucosa, faeces, blood, semen

Table VIII. Risk factors for infections

Risk of infections	Example
Primary or secondary immune deficiency	SCID, HIV
Immune suppressive treatment	TBI, chemotherapy
Immune modulation	Immune modulatory agents other than TBI and chemotherapy, or in significantly lower doses
Young age	pre-immune children, typically < 5 yrs
Old age	>60 yrs
Predisposing diseases	Diabetes Mellitus
Disability	Immobilization
Intensive contact	Military personnel, day care centers, nursing homes, kindergarten, dormitories
Low socio-economic status	Lack of vaccination, malnutrition
Other	Stress, nutrition, alcoholism

Table IX. Adenovirus species, serotypes and receptors

Species	Serotype(s)	Receptor[177,564]	Features [486]
A	12,18,31	CAR	Pneumonia, enteritis
B1	3,7, 16,21,50	sBAR CD46	Hemorrhagic cystitis
B2	11, 14, 34, 35	sBAR, CD46 sBAR CD46	Hemorrhagic cystitis
C	1,2,5,6	CAR	Hepatitis, pneumonia, disseminated, high risk post-SCTx
D	9,10,13,15,17,20,22-30, 32,33,36,38,39,42-49,51 8, 19a,37, 53, 54	CAR CAR sialic acid	Eye, gastrointestinal tract
E	4	CAR	Respiratory tract
F	40,41	CAR	Gastrointestinal tract
G (proposed)	52		Gastrointestinal tract [565]

Table X. AAV serotypes and receptors

Serotype	Host	Receptors	Organ tropism
AAV-1	Human/non-human primates	Sialic acid	Muscle, CNS, liver
AAV-2	Human	HSPG, heparin, FGFR1, HGF, integrins $\alpha_v\beta_5$, $\alpha_5\beta_1$	Kidney, liver, muscle, lung, CNS
AAV-3	Human	HSPG, heparin	
AAV-4	Non-human primates	Sialic acid	CNS (eye)
AAV-5	Human	Sialic acid, PDGF-R	CNS (eye)
AAV-6	Human and non-human primates	HSPG, sialic acid, heparin	Skeletal muscle, liver
AAV-7	Human and non-human primates		Skeletal muscle
AAV-8	Human and non-human primates		Heart, liver, skeletal muscle, pancreas
AAV-9	Human and non-human primates		Liver, skeletal muscle, lung
AAV-10	Non-human primates		
AAV-11	Non-human primates		

Table XI. Prevalence and severity of virusinfections in immunocompromised patients

Virus	SCID	AIDS	Cytotoxic agents/ Malignancies[566]	Stem cell / organ transplantion[488,566]
HSV*	++	++	15%	90%
CMV	++	+++	32-58%	3-17%
VZV*	+++	++	2-15%	25-60%
HHV6*, HHV8		+++	++	+
EBV	+++	+	++	+++
Measles	++	+	+	+
AdV*	B	D, B	++	5-29% A, B, C (SCT)[486], B (kidney), C (liver)
HBV, HCV		++		++
Reovirus, rotavirus	++			
Papova virus (HPV, BK, JC)	++	+	++	++, up to 95% in SCT
HIV		+++		+

Adapted from Human Virology by Leslie Collier and John Oxford (1993): +++ very common and often severe;
++ common, moderately severe; + infrequent or mild; +/- rare; * mostly reactivation.

Table XII. Common virus serotypes found in normal and immunocompromised patients

Virus	DNA/ RNA	Commonly used lab strains	Normal population	SCID	SCT	Immunosuppression Organ transplant [488]	AIDS	NAB	Recomb/ Shedding
AdV	ds DNA	2,5	1,2,3,4,5,7, 41 Military: 3,4,21	1,2,3,5,7, 4,1,11,31,34,35	1,2,3,4,5,6,7, 8,11,12,15,21,29,31,34,35	Liver Tx: 1,2,5 (C) Renal Tx: 7,11,34,35 (B)	Gut: 9,17,20,22, 23,26,27,42,51 (D) Urine: 11, 34,35 (B2)	+++	++/++
Dependovirus *AAV *Parvovirus	ss DNA	 AAV-2 Rat H-1, MVM	 2, 1, 5, 6, 8, 9 Human B19			 Human B19		 +	 --/-- --/--
HHV HSV-1 Other	ds DNA	 F, 17+, KOS	 HSV-1, VZV		 HSV-1, CMV, VZV, HHV-6, EBV	 HSV-1, CMV, VZV, HHV-6, EBV, HHV-8	 KSHV	 --	 ?/?
RV	RNA	MLV, HIV-1	None	None	None	HIV	HIV-1, HIV-2	--	?/?
Vaccinia (VACV)	ds DNA	NYCBH WR MVA	NYCBH (after vaccination)	None	None	None	None	+++	--/+
VSV	(-) RNA	Cattle, horse, swine	None	None	None	None	None	--	--/--
Morbilli (Measles)	(-) RNA	Life attenuated Edmonton (Edm)	Wt MV	wt	wt	Wt	wt	++	?/--
Rubella (Mumps)	(-) RNA	Life attenuated Jeryl-Lynn		None	None	None	None	++	?/--
NDV	(-) RNA	73-T, Ulster	None	None	None	None	None	--	
Influenza A	(-) RNA	Influenza A/PR8 (H1N1)	A (H1N2, H3N2), B			Influenza		+/-	+/+
Enterovirus	(+) RNA	Coxsackie Virus A21 (CVA21)	CVA21					+	+/?
Poliovirus	(+) RNA	Life attenuated Sabin/PV1-RIPO	None					+	?/?
Reovirus	ds RNA	Type I Lang, Type 2 Jones, Type 3 Dearing	Type I Lang, Type 2 Jones, Type 3 Dearing					++	+/+
Hepatitis	ds DNA					HBV, HCV	HBV, HCV		

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